

TRENDS IN COMPARATIVE ENDOCRINOLOGY AND NEUROBIOLOGY

EDITED BY : Hubert Vaudry and Olivier Kah

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TRENDS IN COMPARATIVE ENDOCRINOLOGY AND NEUROBIOLOGY

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White stork (*Ciconia ciconia*) mating in their nest on top of a building in Andalusia (Spain). Comparative endocrinology allows understanding the complex hormonal interactions underlying sexual maturation and behavior in a wide range of animals.

Image: Olivier Kah.

The comparative approach takes advantage of the biological diversity to select the most appropriate model organism to tackle a scientific question. Comparisons between the endocrine and nervous systems across species have yielded major breakthroughs in endocrinology and neurobiology. For instance: a number of mammalian peptide hormones and neuropeptides have been originally identified in fish or amphibians; studies conducted in a sea slug founded the cellular and molecular basis of learning and memory; observations of neurogenesis in the forebrain of

songbirds led to the discovery of adult neurogenesis in the mammalian brain. These examples illustrate the remarkable contribution of the comparative approach for the advancement of neuroendocrinological concepts.

The present e-book is a unique collection of research articles and reviews that provide a representative overview of the latest developments in comparative endocrinology and neurobiology.

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Editorial: Trends in Comparative Endocrinology and Neurobiology

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Keywords: peptide hormones and neuropeptides, biological rhythms, reproduction, endocrine disrupters, behavior

Editorial on the Research Topic

Trends in Comparative Endocrinology and Neurobiology

Neil Campbell once described evolution as the “overarching theme of biology.” Indeed, many comparative studies have significantly contributed to major discoveries in biological sciences. This is particularly true in the field of comparative endocrinology and neurobiology. For instance, the concept of neurosecretion has been established from studies conducted in invertebrates and fish (1, 2). Similarly, a number of peptide hormones and neuropeptides [e.g., melanin-concentrating hormone, urocortin, urotensin II (UII), glucagon-like peptide-1, stanniocalcin, etc.] have been identified in non-mammalian vertebrates before being “re-discovered” in mammals including humans (3–12). These breakthroughs bear witness to the power of the comparative approach to the current knowledge in endocrinology and neurobiology. They certainly support the famous assertion by Theodosius Dobzhansky that “Nothing in biology makes sense except in the light of evolution” (13).

This research topic is a compilation of contributions stemming from the 27th Conference of the European Comparative Endocrinologists (CECE2014) held in Rennes, France, that illustrates various facets of current comparative endocrinology and neurobiology investigations.

The neuropeptide allatotropin (AT), which was originally isolated in the central nervous system of the tobacco hornworm, was named after its ability to stimulate juvenile hormone (JH) biosynthesis from the corpora allata. Lismont et al. have characterized the cDNA sequence of AT and its receptor in the desert locust *Schistocerca gregaria*. Expression of the receptor cDNA in a mammalian cell line reveals that AT causes Ca²⁺ mobilization and stimulates cAMP production. On locust isolated organs, AT stimulates gut contraction and JH biosynthesis.

Juvenile hormone is an ester of farnesol, a cholesterol precursor found in all eukaryotes. Since, in insects, JH maintains larvae in a juvenile state, De Loof et al. raise the provocative question as to whether farnesol-like endogenous compounds act as anti-aging factors in vertebrates. Indeed, tissue extracts from mammalian organs do display JH activity in insects. In addition, farnesol, its precursor, and/or its metabolites occur in various mouse tissues. Thus, the authors elaborate a hypothesis on the possible roles of these compounds in vertebrates.

A large proportion of regulatory peptides are α -amidated. SALFamides are a family of myorelaxant neuropeptides that occur in echinoderms. Elphick et al. have characterized the SALFamide precursors in representative species of the five echinoderm classes. From these data, they can propose different scenarios regarding the phylogenetic history of the SALFamide neuropeptide family.

The neurohypophysial nonapeptides arginine vasopressin (AVP) and oxytocin are the mammalian representatives of a large family of neuropeptides that appeared very early during evolution. Banerjee et al. have cloned the cDNA encoding the precursors of vasotocin and isotocin, the AVP, and oxytocin counterparts, in two air-breathing catfish. The two precursor genes are expressed both in the brain and the follicular envelope of the ovary and display higher expression during the

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reproductive season, suggesting a possible role in the control of ovarian activity.

Three families of G protein-coupled receptors (GPCRs) have been identified in mammals. There is now clear evidence that oligomerization of GPCRs impacts their trafficking, ligand binding, and signaling. While intrafamily oligomer formation has been extensively studied, interfamily oligomerization is less documented. In their review, Ng and Chow describe the different methods applied to study oligomerization of GPCRs and summarize the current knowledge concerning interfamily GPCR heteromerization.

Parathyroid hormone (PTH) is a peptide that plays a crucial role in the regulation of calcium homeostasis. PTH belongs to a family of peptides that includes PTH-related peptide and the tuberoinfundibular peptide of 39 residues (TIP39 also called PTH2). On et al. describe the molecular evolution of these peptides and their receptors in vertebrates. Their review indicates that these peptide–receptor systems appeared early during evolution, possibly in invertebrates.

In mammals, the timing of ovulation is crucial for the success of reproduction: plasma estradiol level acts as an indicator of oocyte maturation while peptidergic neurons within the supra-chiasmatic nucleus signal the time of the day and synchronize female fertility with the period of maximal activity and sexual motivation. Simonneaux and Bahougne summarize the literature pertaining to the inhibitory and stimulatory actions of estradiol on the reproductive axis and describe the roles of AVP and vasoactive intestinal peptide in the timing of the luteinizing hormone (LH) surge. They also review the contribution of peripheral clocks within the reproductive organs in the timing of female reproduction. Finally, they discuss the impact of shift work on female fertility.

In photoperiodic mammals, the duration of melatonin secretion during the night regulates seasonal changes of physiological functions. Chakir et al. show that, in the Syrian hamster, nocturnal melatonin exerts chronomodulatory effects on cortisol, leptin, insulin, and glucose daily rhythms. Their study thus provides important information regarding the role of melatonin in the circadian control of energy metabolism.

Besides the pineal gland, melatonin is synthesized by several organs including the retina and gastrointestinal tract. Mukherjee and Maitra review the physiological significance of gut-derived melatonin in vertebrates. While pineal-derived melatonin acts mainly as a neurohormone/neuromodulator, gut melatonin appears to act primarily locally as an autocrine/paracrine factor.

Domestic animals are fruitful models for comparative endocrinologists and neurobiologists. In spring, ewes normally do not ovulate, but exposure to a ram or to ram odor can induce an LH surge and ovulation. This pheromone response, known as the “ram effect,” can be used to compact lambing in a herd. Fabre-Nys et al. describe the neuroanatomical pathways and neurochemical mechanisms underlying the ram effect. They also show that the ram effect is a relevant model in which to address more general questions regarding the neuronal and endocrine regulation of the hypothalamic–pituitary–gonadal axis.

Kisspeptin has recently emerged as a major player of the neuroendocrine control of reproduction and puberty. Skrapits et al.

review the literature regarding the occurrence of various neurotransmitters and neuropeptides in kisspeptin neurons both in laboratory animals and in humans. Their data reveal that human kisspeptin neurons express far more neuropeptides than those of laboratory species, suggesting that the fine control of fertility differs substantially between primates and non-primates.

Age-related dysfunctions in the endocrine system are usually associated with a decline of cognitive functions and a higher risk of neurodegenerative diseases. Blair et al. review the evidence that changes in gonadal steroids, LH, and even sex hormone-binding globulin can be involved in learning and memory impairment and development of Alzheimer’s disease. These data could lead to novel therapeutic strategies for the treatment of neurodegenerative disorders.

Aromatase catalyzes the conversion of testosterone into 17 β -estradiol, and the inactivation of 17 β -estradiol into catechol estrogens. Charlier et al. summarize the data showing that aromatase activity can be rapidly modified through phosphorylation/dephosphorylation processes. The fine tuning of aromatase phosphorylation in specific brain areas may thus account for the multiple physiological and behavioral effects of estrogens.

The peptide hormones adrenocorticotropin and melanocyte-stimulating hormones act through five GPCRs collectively called melanocortin receptors. The functional expression of this family of receptors is regulated by two melanocortin receptor accessory proteins, MRAP1 and MRAP2. Jackson et al. elaborate on the impact of MRAP deletions in adrenal deficiency and obesity, as revealed by animal model studies and human mutations.

The neuropeptide UII is a potent vasoconstrictor. UII belongs to a family of peptides that also includes three UII-related peptides, i.e., URP, URP1, and URP2. Vanegas et al. have compared the central and peripheral actions of UII, URP1 and URP2 on cardioventilatory and locomotor functions in unanesthetized trout. They show that intracerebroventricular injection of each of the three neuropeptides increases ventilation, blood pressure, heart rate, and locomotion with different potencies, whereas intraarterial administration of only a high dose of UII and URP1 (but not URP2) stimulates ventilation and locomotor activity.

In invertebrates, as in vertebrates, serotonin (5-HT) exerts multiple behavioral and neurophysiological actions. In the insect *Rhodnius prolixus*, the chief Chagas disease vector, 5-HT acts as a diuretic hormone that regulates hemolymph osmolarity after its dramatic feeding bout. Paluzzi et al. have cloned the 5-HT_{2b} receptor cDNA in *R. prolixus* and studied its pharmacological profile on transfected cells and isolated organs.

In mammals, transthyretin (TTR) acts as a thyroxine (T₄)-binding protein whereas, in sub-mammalian vertebrates, TTR binds preferably the active form of thyroid hormones, triiodothyronine (T₃). Richardson reviews the evolution of the TTR gene and describes how subtle changes in the protein structure result in the switch of the ligand from T₃ to T₄.

The glycoprotein hormone (GPH) family encompasses LH, follicle-stimulating hormone, thyroid-stimulating hormone, and chorionic gonadotropin. Although GPHs are present only in vertebrates, related GPH subunit ancestor genes have been identified in most vertebrates phyla. Cahoreau et al. provide an

extensive and critical look at the structure and function of GPHs and their receptors.

5'AMP-activated protein kinase (AMPK) is a protein kinase that is activated by ATP deficiency. AMPK is primarily expressed in the liver and muscle where it acts as an energy sensor. In invertebrates and vertebrates, AMPK is also expressed in the gonads. Bertoldo et al. take a broad comparative view to examine the role of AMPK in the interplay between the regulation of energy homeostasis and reproductive functions.

Steroid hormones influence the fertility, development, and survival of parasites; reciprocally, parasite infection often affects plasma steroid levels in the host. Romano et al. provide evidence that a number of parasites express several steroidogenic enzymes and can synthesize various steroid hormones including ecdysteroids, sex steroids, and corticosteroids.

Endocrine-disrupting chemicals (EDCs) are pharmaceutical or environmental compounds that mimic or impair the action of hormones. EDCs can thus affect a number of physiological and behavioral actions of hormones in animals and humans. Grimaldi et al. compare the effects of EDCs, at the cellular and molecular levels, on human and zebrafish estrogen and peroxisome proliferator-activated γ receptors. They point out that the transcriptional activities of EDCs on human and zebrafish nuclear receptors exhibit marked differences. They conclude that caution

should be exercised regarding extrapolation of EDC screening tests in zebrafish models toward hazard assessment for human physiopathology. Rosenfeld focuses on the effect of bisphenol A and phthalate exposure on parental behavior and the impact of developmental exposure to these EDCs on social behavior. They provide convincing evidence that these chemicals alter the organizational and activational programming the brain.

It is our hope that this research topic will become a major set of references for comparative endocrinologists and neurobiologists and will raise the interest of other scientists who are not (yet) involved in this fertile research domain.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Molecular cloning, sequencing and tissue expression of vasotocin and isotocin precursor genes from Ostariophysian catfishes: phylogeny and evolutionary considerations in teleosts

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Basic and neutral neurohypophyseal (NH) nonapeptides have evolved from vasotocin (VT) by a gene duplication at the base of the gnathostome lineage. In teleosts, VT and IT are the basic and neutral peptides, respectively. In the present study, VT and IT precursor genes of *Heteropneustes fossilis* and *Clarias batrachus* (Siluriformes, Ostariophysi) were cloned and sequenced. The channel catfish *Ictalurus punctatus* NH precursor sequences were obtained from EST database. The catfish NH sequences were used along with the available Acanthopterygii and other vertebrate NH precursor sequences to draw phylogenetic inference on the evolutionary history of the teleost NH peptides. Synteny analysis of the NH gene loci in various teleost species was done to complement the phylogenetic analysis. In *H. fossilis*, the NH transcripts were also sequenced from the ovary. The cloned genes and the deduced precursor proteins showed conserved characteristics of the NH nonapeptide precursors. The genes are expressed in brain and ovary (follicular envelope) of *H. fossilis* with higher transcript abundance in the brain. The addition of the catfish sequences in the phylogenetic analysis revealed that the VT and IT precursors of the species-rich superorders of teleosts have a distinct phylogenetic history with the Acanthopterygii VT and IT precursors sharing a less evolutionary distance and the Ostariophysi VT and IT having a greater evolutionary distance. The genomic location of VT and IT precursors, and synteny analysis of the NH loci lend support to the phylogenetic inference and suggest a footprint of fish-specific whole genome duplication (3R) and subsequent diploidization in the NH loci. The VT and IT precursor genes are most likely lineage-specific paralogs resulting from differential losses of the 3R NH paralogs in the two superorders. The independent yet consistent retention of VT and IT in the two superorders might be directed by a stringent ligand-receptor selectivity.

Keywords: catfish, cloning, phylogeny, vasotocin-isotocin precursors, synteny analysis

Introduction

The neurohypophyseal (NH) nonapeptides are an evolutionarily old family which is believed to have originated much before the vertebrates, as one of the first neurotransmitters in the archetype metazoan nervous system (Grimmelikhuijzen et al., 2002). Starting from the most primitive metazoan- the cnidarians, the presence of nonapeptide homologs has been documented in several nonvertebrate phyla (Grimmelikhuijzen et al., 1982; Cruz et al., 1987; Proux et al., 1987; Satake et al., 1999; Takuwa-Kwodo et al., 2003; Kawada et al., 2008; Stafflinger et al., 2008). In vertebrates, studies are relatively extensive and the nonapeptide precursor genes have been cloned from several vertebrate groups: cyclostomes (Lane et al., 1988; Suzuki et al., 1995; Gwee et al., 2009), cartilaginous fish (Hyodo et al., 2004; Gwee et al., 2009), teleosts (Heierhorst et al., 1989; Morley et al., 1990; Hyodo et al., 1991; Suzuki et al., 1992; Venkatesh and Brenner, 1995; Warne et al., 2000), lungfishes (Hyodo et al., 1997), coelacanth (Gwee et al., 2008), amphibians (Nojiri et al., 1987; Searcy et al., 2009), reptiles (Montefiano et al., 2001), and birds (Hamann et al., 1992). This has complemented earlier works of characterization of peptide principles of the neurohypophysis by chromatographic methods and has provided useful insights into the distribution of the peptides in different vertebrate species, their precursor structure and evolution. The nonapeptides are synthesized as part of a large precursor molecule that undergo post-translational processing to release the nine amino acid hormone with two half cysteine residues at the first and sixth positions forming a disulphide bridge, and a cysteine- rich protein called neurophysin (Acher, 1996). Neurophysin aids in proper folding of the precursor and its sorting into secretory vesicles (de Bree et al., 2000). In vertebrates, about 15 nonapeptides are known till date. They are classified into two families on the basis of the amino acid at the 8th position, as the basic and neutral peptide families. Each vertebrate possesses at least two peptides, one each from the two families except cyclostomes, where only the basic peptide representative vasotocin (VT) is documented (Gwee et al., 2009). This has led to a widely accepted theory that the VT precursor gene is the ancestor of all vertebrate nonapeptide precursor genes, and has given rise to the two lineages following an event of duplication early in the vertebrate evolution probably at the base of the gnathostome lineage. Evolutionarily too, the two peptide families follow distinct lineages with VT representing the common basic peptide in all nonmammalian vertebrates to be replaced by vasopressin (VP) in mammals, while isotocin (IT) is the neutral peptide in teleosts, mesotocin (MT) in lobe-finned fishes and noneutherian tetrapods, and oxytocin (OT) in eutherians. In elasmobranchs, a host of different neutral peptide homologs has been reported which is in contrast with the remarkable stability in the type and numbers of nonapeptides in any other group of Vertebrata (Acher, 1996). Presence of more than two neurohypophyseal peptides has been reported from groups like elasmobranchs, amphibians, and metatherians (Chauvet et al., 1983, 1984, 1985; Rouille et al., 1988, 1989; Parry et al., 2000; Hyodo et al., 2004). In teleosts, VT and IT have been reported as the basic and neutral hormone representatives, respectively. Larhammar et al. (2009) emphasized the need for

simplifying the nomenclature with the original names VP for the basic peptides and OT for all neutral peptides.

The two evolutionary lines of basic and neutral nonapeptides across vertebrates represent a contrasting picture in the frequency of substitutions in the 9 amino acid (aa) hormone. In the basic lineage, there have been lesser substitutions than that in the neutral line, as evident from the distribution of the peptides in vertebrates. In fact, the number of cognate receptors for the two peptide families is also contrasting. While the stable basic nonapeptide line has a diverse repertoire of at least five types of cognate receptors (V1A, V1B, V2A, V2B, and V2C), the neutral nonapeptide series has a single type (OTR) in all vertebrate groups (Ocampo Daza et al., 2012; Yagamuchi et al., 2012; Lagman et al., 2013). All types of receptors are present in the early gnathostomes, the cartilaginous fish (Yagamuchi et al., 2012) suggesting that the ligand-receptor selectivity might have been established very early in the vertebrate evolution and it is the co-evolution of the hormone with its cognate receptors which has maintained the two families of basic and neutral nonapeptides, and allowed only a basic amino acid substitution at the 8th position in the basic line and a neutral amino acid substitution at the 8th position in the neutral line. While, the ligand- receptor selectivity might have directed the evolution only in the 9 aa hormone part; the larger neurophysin region is not under this evolutionary pressure. Therefore, to know the evolutionary history of the NH precursors especially in the light of recurrent local and global duplication events occurring throughout the vertebrate history, there is a need to do a phylogenetic study over the entire precursor rather than considering the distribution of peptides in different vertebrate groups.

Teleosts had undergone a third round of whole genome duplication, (3R) (Meyer and Schartl, 1999; Christoffels et al., 2004; Jaillon et al., 2004) in addition to the two vertebrate-specific whole genome duplications. After the 3R in teleosts, there was diploidization by the loss of 3R paralogs. These losses of the 3R paralogs were different in various groups of teleosts (Garcia de la serrana et al., 2014). Even in such a scenario, the consistency in the number and type of nonapeptides in teleosts is noteworthy. However, the teleost NH precursors available in the public database are mostly sequences from the species-rich superorder Acanthopterygii while only few nonapeptide precursor sequences from the other species-rich group, the superorder Ostariophysi are available. The Acanthopterygii and Ostariophysi have undergone independent adaptive radiations (Santini et al., 2009), and are known to have suffered differential losses of 3R paralogs. Therefore, it is pertinent to have sequence information from more species representing all groups in Teleostei, particularly the superorder Ostariophysi to understand the evolution of the NH precursor genes in teleosts.

Previous studies from our laboratory have shown a well-developed and highly organized VT system in the Asian air-breathing catfish *Heteropneustes fossilis*. VT was characterized in both brain and ovary of the catfish with seasonal changes (Singh and Joy, 2008), and has been shown to be involved in reproductive functions such as steroidogenesis, oocyte maturation, oocyte hydration, ovulation and prostaglandin secretion (Singh and Joy, 2009b, 2010, 2011; Joy and Singh,

2013). IT is less effective in influencing these events. Three VT receptors have been cloned from the catfish V1a1 (accession no.KF434615.1), V1a2 (accession no.KF434616.1), and V2A (accession no.KF434617.1), which display a wide tissue distribution (Rawat et al., 2015). All the three receptors are expressed in the brain and ovary, which are the two major sites of VT production.

The catfish *H. fossilis* holds an important phylogenetic position as it is believed to be the sole survivor of an old clade that went extinct at the K-T (Cretaceous-Tertiary transition period) boundary (Jansen et al., 2006) when a major extinction event took place due to severe volcanic activity (Deccan traps) in the Asian region. This might have severely polluted and deoxygenated the inland water bodies and only fish with air-breathing apparatus survived. Air-breathing habit also demands a shift in osmoregulation and fluid homeostasis in which apart from other systems, the NH nonapeptides play a key role. In view of the important taxonomic and phylogenetic position of *H. fossilis* and its air-breathing habit, molecular characterization of the NH peptide genes as a follow on of earlier anatomical and physiological studies on VT has been attempted in the present work. The sequence information was also extended to *Clarias batrachus*, another air-breathing catfish, and used in the phylogenetic analysis. The catfish NH precursor sequence information from the present study will add to the information pool of teleost nonapeptides from a group other than the superorder Acanthopterygii and allow discussing the phylogeny of the vertebrate NH nonapeptides in the light of 3R and subsequent diploidization in teleosts. A tissue distribution profile for these genes was also carried out since VT was described in the catfish ovary earlier.

Materials and Methods

Animal Collection and Acclimation

Adult *H. fossilis* (40–50 g) were collected from local fish markets in Varanasi. They were maintained in the laboratory for 48 h under natural photoperiod (13.0 L: 11.0 D) and temperature ($25 \pm 2^\circ\text{C}$) to overcome stress due to transportation and fed daily with goat liver *ad libitum*. Adult *C. batrachus* were obtained in the resting phase for the cloning work. The fish were weighed and sacrificed by decapitation. All experiments were performed in accordance with the guidelines of the Animal Ethics Committee, Banaras Hindu University, Varanasi.

Chemicals and Reagents

Guanidine thiocyanate—phenol solution (Qiagen), Revert-Aid H Minus First Strand cDNA Synthesis Kit (Fermentas), 2X PCR Master Mix (Fermentas), DNase (Ambion) and veriquest SYBR green qPCR master mix (affymetrix) were purchased through local suppliers. Agarose, Tris base, Glacial acetic acid, EDTA-Na₂ and other chemicals were of molecular grade, purchased from E-Merck, India. LB broth, LB Agar, ampicillin, X-Gal and IPTG were purchased from Himedia, India. Hyaluronidase type IV was purchased from Sigma-Aldrich, India. The primers used were synthesized by Integrated DNA Technology, India.

Cloning of VT and IT genes from *H. fossilis* and *C. batrachus*

For cloning of VT, an approach of first isolating partial VT cDNA using degenerate PCR (polymerase chain reaction) followed by 3' and 5' Rapid Amplification of cDNA ends (RACE) to obtain the full length VT cDNA sequence was applied. Total RNA from the brain of acclimatized female catfish in the resting phase of the reproductive cycle was prepared by the single-step method of RNA isolation (Chomczynski and Sacchi, 1987) by acid guanidium thiocyanate-phenol-chloroform extraction using Qiazol (Qiagen) as the monophasic lysis buffer. Five μg total RNA was reverse-transcribed using random hexamer primers and Revert Aid M-MuLV reverse transcriptase in a 20 μL reaction volume (first strand cDNA synthesis kit, Fermentas) using the manufacturer's protocol. One μL of the resulting cDNA was used to amplify partial VT cDNA in 25 μL reaction volume using 2 X PCR master mix (Fermentas) and VT degenerate forward primer and VT degenerate reverse primer in a cycling condition of one cycle of 95°C , 5 min; then 35 cycles of 95°C , 30 s; 57°C , 30 s; 72°C , 1 min; and a final 7-min elongation at 72°C . The degenerate primers were designed using the software iCODEHOP (Consensus-DEgenerate Hybrid Oligonucleotide Primers), in which teleost VT precursor sequences were used as inputs (Table 1). The resulting 145 bp partial VT amplicon was purified using Nucleo-pore PCR clean-up gel extraction kit (Genetix) and sequenced taking services of Eurofin genomics, Bangalore, India. For obtaining full length sequence, 3' RACE was done for which total RNA from the brain was reverse-transcribed using oligo dT anchor primer and Revert Aid M-MuLV reverse transcriptase. One μL of the resulting cDNA was amplified using a VT H FP (corresponding to the hormone moiety), designed from the partial sequence and anchor primer to obtain a 700 bp 3' RACE amplicon, which was cloned and sequenced. This sequence was utilized for designing two gene-specific nested reverse primers to do 5' RACE. Total RNA from the brain was reverse-transcribed using the outer gene specific VT RP and Revert Aid M-MuLV reverse transcriptase. The resulting cDNA was purified using the Nucleo-pore PCR clean-up gel extraction kit (Genetix) to remove residual dNTPs. The purified cDNA was dA tailed using dATP and terminal transferase. The dA tailed cDNA was used for PCR amplification using the nested RP 2 primer and oligo dT anchor primer. The 5' RACE amplicon was purified, cloned and sequenced. Full length VT cDNA sequence was submitted to the GenBank with the accession no. JX035928.1

For cloning IT precursor cDNA, total RNA from the brain was reverse-transcribed using an oligo dT anchor primer and Revert Aid M-MuLV reverse transcriptase. One μL of the resulting cDNA was PCR amplified using a sense or forward primer IT H FP designed from the IT hormone moiety and anchor primer, following the 3' RACE protocol. The resulting 700 bp amplicon was sequenced taking services from Eurofin genomics. To get the 5' end of the precursor cDNA, 5' RACE was done. Total RNA from the brain was reverse-transcribed using an outer reverse or antisense primer, RP1 (designed over a conserved region of the neurohypophyseal peptide neurophysin) and Revert Aid M-MuLV reverse transcriptase. The resulting cDNA was

TABLE 1 | List of primers used for cloning and qPCR of vasotocin and isotocin precursors.

| Primers | Sequences |
|------------------------|--------------------------------------|
| VT d FP | TCCGCTTGTTACATCCARAAYTGYCC |
| VT d RP | ACATCCCAGTCCCTCTCCRCARCDAT |
| VT H FP | GTTACATCCAGAACTGCCCCAGA |
| VT FP | TGTTACATCCAGAACTGCCCCAGA |
| VT RP | CAGCCCAGTCTTCTCCACAGCA |
| RP1 | TTTATCTCCAGACCGCAAG |
| RP2 | CAGGACGCAAGACATACAC |
| VT UTR FP | GTCCAGTGAGAGACAGACCTCCGG |
| IT H FP | ACATCTCCAAGTGTCCATC |
| IT UTR FP | CATCAGCTACTGAAGCTACTGATTCGT |
| Oligo dT anchor primer | GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTT |
| Anchor primer | GACCACGCGTATCGATGTCGAC |
| IT FP | TCAATCTTCTGCATGCTGTGTCT |
| IT RP | CACACGCCATGCACTGTCTATTG |
| β actin FP | TGGCCGTGACCTGACTGAC |
| β actin RP | CCTGCTCAAAGTCAAGAGCGAC |

purified and dA tailed. The dA tailed cDNA was used for PCR amplification using an overlapping and nested antisense primer RP2 and oligodT anchor primer. The resulting 200 bp 5' RACE amplicon was ligated into pGEMT vector (promega kit), following the manufacturer's protocol and transformed into the *E. coli* DH5 α competent cells. Transformed cells were plated into LB Agar solid medium containing ampicillin, X- Gal and IPTG to select positive transformants and blue-white screening was done for recombinant colonies. Plasmids from white colonies (colonies transformed with recombinant plasmids) were extracted by alkaline lysis method and sequenced. Sequence information from the IT precursor containing inserts (as known by *in silico* translation of the insert sequences) was used to design a sense primer from the 5' UTR region (IT UTR FP), followed by 3' RACE with this primer to obtain the entire IT precursor sequence, which was submitted to the GenBank with the accession no. JX669009.1.

Partial sequences of *C. batrachus* VT and IT precursors were obtained by 3' RACE with the forward primers VT H FP and IT H FP (the same used for *H. fossilis*), to obtain a sequence read from the hormone region till the 3' untranslated region. The primer details are given (Table 1).

Sequencing of VT and IT Transcripts from Ovary

Since the VT and IT transcripts are less abundant in the ovarian follicles, a semi-nested 3' RACE was done to get a single, sharp amplification of the gene transcripts. Briefly, it included a primary PCR using VT UTR FP, IT UTR FP spanning the 5' UTR region of VT and IT genes, respectively and anchor primer to amplify 1 μ L of follicular cDNA (made by reverse transcription of RNA using oligodT anchor primer), followed by a secondary PCR using VT H FP, IT H FP and anchor primer to amplify 1 μ L of the primary PCR product. The amplicons from the secondary PCR was purified and sequenced.

Data Mining for Phylogenetic Analysis

The NH nonapeptide precursor sequences of species representing most vertebrate classes were tried to be included in the phylogenetic analysis. The sequences were procured from the GenBank. Details of the sequences of the species with accession numbers are given in Supplementary Table 1. For procuring the NH precursors of *Ictalurus punctatus*, a BLAST search against the *Ictalurus* EST database (Lu et al., 2010) was done using the *H. fossilis* VT and IT sequences as queries. The identities of the putative VT and IT precursor mRNAs obtained were confirmed by *in silico* translation of the sequences. The spotted gar *Lepisosteus oculatus*, Holostei is a basal actinopterygian species representing a pre 3R genome. The neurohypophyseal nonapeptide precursor sequences of this species were procured by BLAST searches in its Nucleotide database and analyzed.

Phylogenetic Analysis

Phylogenetic trees were constructed using the Neighbor- Joining (NJ) and Maximum-Likelihood (ML) method. Evolutionary distances were computed using the Poisson correction method for rate of amino acid substitution. All phylogenetic studies were done using the software MEGA 6 (Tamura et al., 2013). In addition to the calculation of evolutionary distance of precursor sequences within and between species, the sequences were grouped according to the taxonomic units for calculation of evolutionary distance between groups (Table 2). Keeping in view of the enormous species diversity, the teleost sequences of the two superorders Acanthopterygii and Ostariophysi were independently treated. Multiple sequence alignment used for the tree construction and distance calculation was the same and was done in MEGA6 using Clustal W parameters. Gonnet protein weight matrix with gap opening and gap extension penalties of 10 and 0.2 respectively was used for the alignment. The alignment is provided in a Supplementary data sheet 1.

Analysis of VT and IT Genomic Location and Synteny Analysis

The nonapeptide gene-containing chromosome blocks of spotted gar and representative teleost species were obtained from the NCBI and Ensembl genome browser. Fugu, medaka, tilapia, and stickleback belong to the teleost superorder Acanthopterygii, and zebrafish and cavefish belong to the superorder Ostariophysi. The selection of the chromosome blocks from Ensembl database was based both on searching the genome assemblies for the nonapeptide genes by named searches as well as BLAST searches using teleost nonapeptides as queries. This is to ensure that nonapeptide loci are obtained even in the species where the genes are not annotated. The chromosome blocks were obtained from the NCBI database mostly by navigating the genomic context of the genes. Blocks obtained from both Ensembl and NCBI were tallied for each species and a consensus assembly of genes on the blocks was arrived at by filling in for the nonannotated genes in one of the databases from information obtained from the other. Apart from the nonapeptide genes, the assemblies were also searched for the genes linked with the nonapeptide genes and the additional chromosomal blocks harboring these genes (paralogous to the linked genes), if distinct from the

TABLE 2 | Details of the taxonomic groups and the species included in each for the phylogenetic distance calculation between groups.

| S. No | Taxonomic group | Species |
|-------|---|--|
| 1 | Cyclostome VT | <i>Lethenteron camtschaticum</i> , <i>Eptatretus burgeri</i> |
| 2 | Acanthopterygii VT | <i>Thalassoma bifasciatum</i> , <i>Parajulis poecilepterus</i> , <i>Halichoeres trimaculatus</i> , <i>Sparus aurata</i> , <i>Takifugu rubripes</i> , <i>Platichthys flesus</i> , <i>Epinephelus coioides</i> , <i>Amphiprion melanopus</i> , <i>Oryzias latipes</i> , <i>Cyprinodon variegatus</i> , <i>Haplochromis burtoni</i> |
| 3 | Ostariophysi VT | <i>Heteopneustes fossilis</i> , <i>Clarias batrachus</i> and <i>Ictalurus punctatus</i> , <i>Danio rerio</i> , <i>Astyanax mexicanus</i> (LOC103042813, LOC103030472) |
| 5 | Cartilaginous fish VT | <i>Triakis scyllium</i> , <i>Callorhynchus milli</i> |
| 6 | Lungfish VT | <i>Neoceratodus forsteri</i> , <i>Protopterus annectens</i> |
| 7 | Coelacanth VT | <i>Latimeria menadoensis</i> |
| 8 | Acanthopterygii IT | <i>Platichthys flesus</i> , <i>Sparus aurata</i> , <i>Halichoeres trimaculatus</i> , <i>Parajulis poecilepterus</i> , <i>Takifugu rubripes</i> , <i>Oryzias latipes</i> , <i>Xiphophorus maculatus</i> , <i>Amphiprion melanopus</i> , <i>Haplochromis burtoni</i> |
| 8 | Ostariophysi IT | <i>Heteopneustes fossilis</i> , <i>Clarias batrachus</i> , <i>Ictalurus punctatus</i> , <i>Danio rerio</i> , <i>Asatyanax mexicanus</i> (LOC103043649, LOC103044969) |
| 10 | Cartilaginous fish neutral hormone precursors | <i>Triakis scyllium phasitocin</i> , <i>Triakis scyllium asvatocin</i> , <i>Torpedo marmorata isotocin</i> , <i>Callorhynchus milli oxytocin</i> |
| 11 | Lungfish MT | <i>Neoceratodus forsteri</i> , <i>Protopterus annectens</i> |
| 12 | Coelacanth MT | <i>Latimeria menadoensis</i> |
| 12 | Amphibian VT | <i>Bufo japonicus</i> , <i>Plethodon shermani</i> , <i>Taricha granulosa</i> , <i>Typhlonectes natans</i> |
| 13 | Amphibian MT | <i>Bufo japonicus</i> , <i>Typhlonectes natans</i> , <i>Taricha granulosa</i> |
| 14 | Reptilian VT | <i>Podarcis siculus</i> |
| 15 | Reptile MT | <i>Podarcis siculus</i> |
| 16 | Avian VT | <i>Taeniopygia guttata</i> , <i>Coturnix coturnix</i> , <i>Gallus gallus</i> |
| 17 | Avian MT | <i>Taeniopygia guttata</i> |
| 18 | Mammalian VP | <i>Homo sapiens</i> , <i>Rattus norvegicus</i> |
| 19 | Mammalian OT | <i>Homo sapiens</i> , <i>Rattus norvegicus</i> |

The accession numbers of the sequences are given in Supplementary Table 1.

nonapeptide loci. Additionally, the loci of the linked genes in human were obtained and used as a reference tetrapod assembly for comparison of conserved synteny.

Analysis of VT and IT Precursor Gene Expression

A two step qPCR was conducted to show tissue and seasonal expression of VT and IT genes. For tissue expression profile, various tissues viz. brain, gills, liver, muscle, kidney, and gonads were collected. For the ovarian tissue sample, the follicles were collected from the post-vitellogenic ovary and the follicular layer was separated from oocyte using hyaluronidase treatment (Mishra and Joy, 2006). Briefly, the protocol included treatment of a batch of about 500 follicles with 0.3% hyaluronidase (Type IV) for 5 min. The isolated follicular envelope (granulosa and theca cells) and the denuded oocytes were used for the expression studies. For seasonal expression, brain and ovary was collected during the different reproductive phases, resting (November–January), preparatory (February–April), pre-spawning (May–June), spawning (July–August) and post-spawning (September–October). About 100 mg tissues were used for extraction of total RNA by the single-step method of RNA isolation. To remove genomic DNA contamination from the preparation, DNAase (Ambion) treatment (2 units/10 µg RNA) was given and subsequently DNAase was heat inactivated at 75°C in presence of EDTA. RNA purity was checked by A₂₆₀/A₂₈₀ ratio. Samples having a ratio above 1.8 were only considered for reverse transcription. Two µg of the total RNA was reverse-transcribed using random hexamer primers and Revert Aid M-MuLV reverse transcriptase in a 20 µL reaction

volume (first strand cDNA synthesis kit, Fermentas) using the manufacturer's protocol. The resulting cDNA was diluted 10 times and 1 µL was used in a PCR reaction of 20 µL containing veriquest SYBR green 2X master mix and VT/IT FP and IT FP/ RP using manufacturer's protocol in an Applied Biosystem 7500 machine with a thermal condition of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The specificity of the PCR product was checked by dissociation curve analysis of the amplicon and was also checked by agarose gel electrophoresis. The relative gene expression in different tissues and across different reproductive seasons was expressed using the comparative CT method with the catfish β-actin (accession number FJ409641.2), used as the endogenous control. The resting phase brain cDNA was taken as the calibrator sample for relative quantity calculation (Livak and Schmittgen, 2001). Each reaction was set up in duplicate and the average CT value was taken for calculation. Graphs were plotted with the mean RQ (relative quantity) values ($2^{-\Delta\Delta CT}$) of five fish and represented as mean ± SEM. The results were analyzed by One-Way ANOVA ($p < 0.001$), followed by Newman-Keuls' test ($p < 0.05$) for statistical significance.

Results

Sequence Analysis of VT and IT Precursors from *H. fossilis*

The isolated full length cDNA of the HfVT precursor is 618 bp long with a cds from 60 to 524 bp and encodes a VT precursor

of 155 amino acids (aa; **Figure 1**; Supplementary Figure 1A). The deduced VT precursor codes for a signal peptide (1–20 aa), a 9 aa hormone moiety (21–29 aa), a 3 aa enzymatic processing site (30–32 aa) and a 122 aa neurophysin (34–155 aa). The central part of the neurophysin (40–118 aa) is highly conserved and belongs to the hormone_5 superfamily (NCBI CDD accession *pfam00184*) of the neurohypophyseal hormones (NCBI CDD accession *smart00003*). There are 14 cysteine residues in this region (42, 45, 53, 59, 60, 66, 76, 86, 93, 100, 106, 107, 112, and 118). The C-terminal part of the neurophysin (119–155 aa) is poorly conserved except for the presence of a leucine-rich region “LLLRLH” (141–146 aa) (**Figure 1**) that is conserved across all vertebrate groups. All these features conform to the typical features of the neurohypophyseal hormones. There is no putative site for N-linked glycosylation in C-terminal domain corresponding to copeptin, making it akin to other teleost neurohypophyseal hormone precursors, unlike lungfish and tetrapod hormone precursors.

The isolated HfIT precursor cDNA is of 708bp long with a cds from 56 to 508 bp and encodes the hormone precursor of 151 aa (**Figure 1**; Supplementary Figure 1B). Similar to the VT precursor, the putative IT precursor encodes for a signal peptide (1–20 aa), a hormone moiety of 9 aa (21–29 aa), enzymatic processing signal (30–32 aa) and a 118 aa neurophysin (34–151 aa). Further, the central part of neurophysin (40–118 bp)

is highly conserved and belongs to the hormone_5 superfamily (NCBI CDD accession *pfam00184*) of the neurohypophyseal hormones (NCBI CDD accession *smart00003*) and there are 14 cysteine residues in this region (42, 45, 53, 59, 60, 66, 76, 86, 93, 100, 106, 107, 112, 118) (**Figure 1**). The presence of an extended C-terminal part homologous to the C-terminal part of the VT precursors and the mammalian vasopressin-associated copeptin makes it similar to other IT precursor but dissimilar to other neutral peptide precursors like MT and OT precursors and other cartilaginous fish neutral hormone precursors which lack an extended C-terminal region. The C-terminal part of the neurophysin (119–151 aa) is poorly conserved except for the presence of a leucine-rich region “LLKLLHL” (138–144 aa), which is a signature of the C-terminal region of the neurohypophyseal hormone precursors. There is neither a dibasic cleavage site nor putative site for N-linked glycosylation in this region similar to the VT precursor.

Sequence Analysis of Other Catfish VT and IT Precursors

The cloned partial sequences of *C. batrachus* (Cb) VT and IT cDNA extend from the N-terminal neurophysin coding region to the 3' UTR (Supplementary Figures 1C,D). The putative precursors, like the *H. fossilis* precursors, have all the essential features of the neurohypophyseal precursor proteins, with a

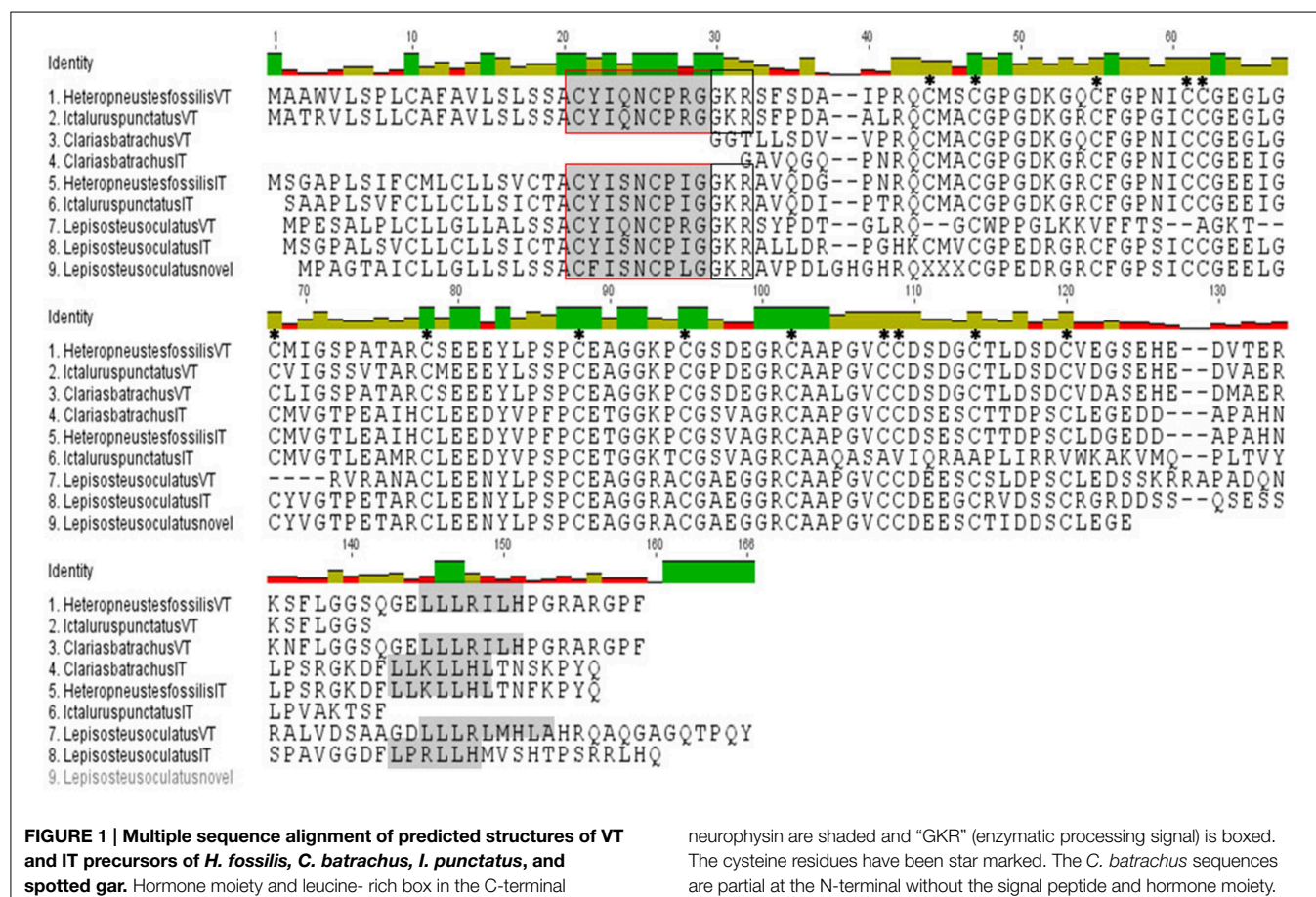


FIGURE 1 | Multiple sequence alignment of predicted structures of VT and IT precursors of *H. fossilis*, *C. batrachus*, *I. punctatus*, and spotted gar. Hormone moiety and leucine-rich box in the C-terminal neurophysin are shaded and “GKR” (enzymatic processing signal) is boxed. The cysteine residues have been star marked. The *C. batrachus* sequences are partial at the N-terminal without the signal peptide and hormone moiety.

central conserved neurophysin having 14 cysteine residues. The poorly conserved C-terminal regions have leucine-rich boxes, “LLLRILH” in the VT precursor and “LLKLLHL” in the IT precursor (**Figure 1**).

In silico translation of the clones, recognized as the neurohypophyseal hormone precursors from the EST database of *I. punctatus* (Ip) by BLAST reveals that they indeed code for VT and IT precursors. The sequence read of the putative VT precursor clone (acc. no. BM495247) starts from 5' UTR region and ends in the C-terminal part of the neurophysin. On the other hand, the putative IT precursor clone (acc. no. BE213165) sequence extends from the signal peptide region to the neurophysin (Supplementary Figures 1E,F and **Figure 1**).

The 3' UTR region of the *H. fossilis* VT gene has a long stretch of 19 CA repeats. The *C. batrachus* VT gene has only 4 CA repeats in the 3' UTR at approximately the same position as the *H. fossilis* VT gene (Supplementary Figures 1A,C).

Analysis of Spotted Gar Neurohypophyseal Nonapeptides

BLAST search revealed that the spotted gar has three neurohypophyseal nonapeptides, VT as the basic nonapeptide (XM 006626529.2) and two nonapeptides in the neutral family, IT (XM 006626499.1) and a novel peptide (XM 006626523.1, 1–366 nt) with a unique hormone “CFISNCPLG” is present. While the spotted gar IT has an extended C-terminal with a leucine-rich core, the novel nonapeptide has a short C-terminal like all other neutral nonapeptide precursors of vertebrates (**Figure 1**).

Tissue and Seasonal Expression of VT and IT Precursor Genes

The nonapeptide genes expressed only in the brain and ovary of the catfish (**Figure 2**). In the ovary, the expression was confined to the follicular envelope and not in oocytes. There was no expression in the gill, liver, kidney, testis and muscle. The VT and IT mRNA expressions were significantly higher in the brain than the ovary (**Figure 2**).

The sequence reads of the semi-nested 3' RACE amplicons of VT and IT in the ovary were partial but good enough to indicate the presence of functional transcripts of the genes in ovarian follicles (not shown). Also, the sequences were similar to the brain transcripts, pointing to the same gene responsible for synthesis of the nonapeptides in both brain and ovary.

VT and IT expressions showed significant seasonal changes both in the brain and ovary (**Figure 3**). In the brain, the VT expression was low in the resting phase and increased in the preparatory, pre-spawning and spawning phases. The expression remained high in the post-spawning phase similar to the preparatory and pre-spawning phases. The expression of IT precursor was similar to that of the VT precursor but it decreased sharply in the post-spawning phase as in the resting phase.

The highest expression was observed in the spawning phase for both the genes (**Figures 3A,B**). In ovary too, the expression of both VT and IT increased in the reproductive phases compared to the resting phase (**Figures 3C,D**). However, while in the spawning phase the expression of the VT precursor was

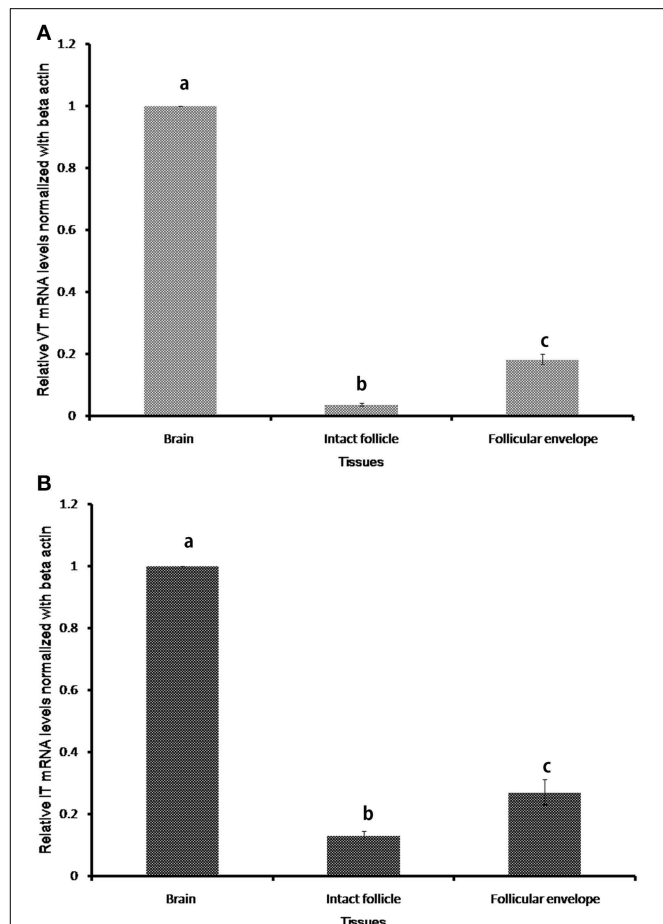


FIGURE 2 | Relative tissue expression levels of VT precursor (A) and IT precursor (B). Graphs were plotted with the mean RQ values ($2^{-\Delta\Delta CT}$) of expression in brain, intact follicle and follicular envelope. The RQ values (mean \pm SEM; $n = 5$ fish each) were calculated with the resting phase brain cDNA as the calibrator. Groups with different alphabets show significant variations in the expression levels.

higher as compared to the preparatory and pre-spawning phases (**Figure 3C**), the IT precursor expression remained equally high in all the reproductive phases (**Figure 3D**).

Evolutionary History of Teleost NH Peptide Precursor Genes

Phylogenetic Analysis

The phylogenetic inference on vertebrate NH peptide precursors was drawn from both tree constructions as well as from the evolutionary distance calculation in between groups. Both the NJ and ML trees are similar and only the ML tree is shown in **Figure 4**. The NH sequences of salmonids and catostomids (teleosts) were included in the initial analysis but were removed from the tree as they did not make a definite cluster probably due to polyploidization that led to two copies of VT and IT genes, and gene conversions (Hyodo et al., 1991; Suzuki et al., 1992). Both the phylogenetic tree and evolutionary distance calculation in between the groups (**Figure 5**) show that the NH precursor paralogs of cartilaginous fish, coelacanth and mammals

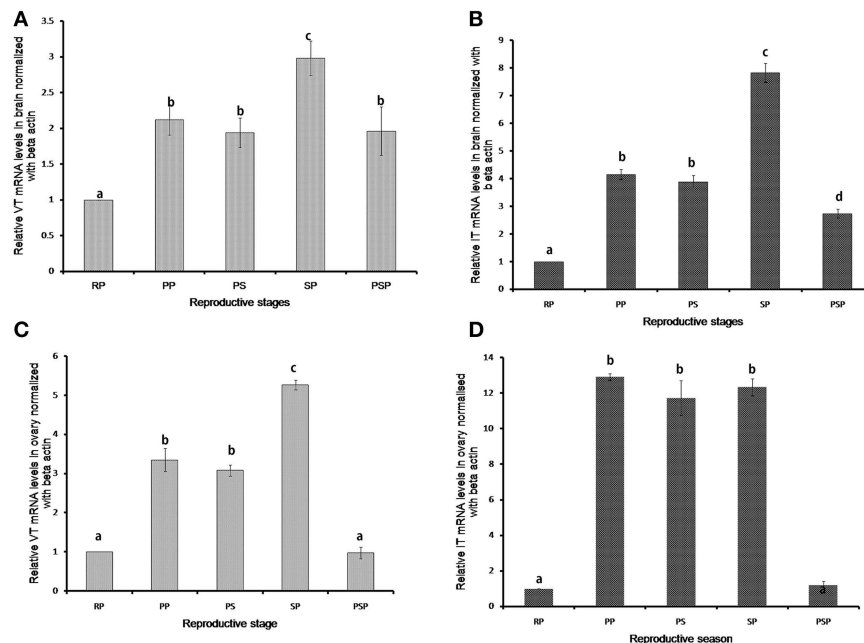


FIGURE 3 | Seasonal expression levels of VT and IT precursors in brain (A,B) and ovary (C,D). The RQ values ($2^{-\Delta\Delta CT}$) were calculated with the resting phase brain or ovary as the calibrator samples (mean \pm SEM; 5

fish each). Groups with different alphabets show significant variations in the expression levels. RP, resting phase; PP, preparatory phase; PS, pre-spawning phase; SP, spawning phase; PSP, post-spawning phase.

are phylogenetically closer than their homologs in other groups. The cartilaginous fish VT and neutral hormone precursors share a distance of 0.350, coelacanth VT and MT precursor share a distance of 0.132, and the mammalian VP and OT precursors share a distance of 0.219. The evolutionary distances of the NH precursors of these groups with that of other vertebrate groups are larger (Figure 5). Amongst the cartilaginous fish, elephant shark VT and OT precursors share a closer phylogenetic relation, as shown by the ML tree.

The phylogenetic tree with the VT precursor of Agnatha as the outgroup also shows that the teleost VT and IT precursors form a cluster with those of the spotted gar (Holostei) while other gnathostome NH precursors form another cluster, which includes the cartilaginous fish VT and neutral hormone precursors, and coelacanth and lungfish VT and MT precursors. However, the bootstrap support for the node separating the teleost NH precursors and the other gnathostome NH precursors is low.

The catfish NH precursors expectedly segregated into their respective clusters, i.e., the HfVT, CbVT, IpVT and HfIT, CbIT, IpIT, making two distinct clusters. Within these clusters, the CbVT, HfVT and CbIT, HfIT are closer to each other than to IpVT and IpIT, respectively. The spotted gar NH precursors made a common cluster; the two neutral NH peptide precursors (IT precursor and the novel peptide precursor) are evolutionarily closer than the VT precursor. The Acanthopterygii VT and IT precursors are phylogenetically closer than their orthologs in the Ostariophysi. The evolutionary distance in between the groups confirms this. The evolutionary distance between the Acanthopterygii VT and IT precursors is only 0.269 while that

between Acanthopterygii VT and Ostariophysi VT precursors is 0.426, and the Acanthopterygii IT and Ostariophysi IT precursors is 0.452. The ostariophysian VT precursor form a cluster having the catfish VT precursors and one of the two VT precursors of cavefish (*Astyanax mexicanus*), while zebrafish (*Danio rerio*) VT precursor does not form part of this cluster. The ostariophysian IT precursors make a cluster that includes the catfish and zebrafish IT precursors, and one of the IT precursors of the cavefish. The other cavefish VT and IT precursors, which do not form a part of the ostariophysian clusters, make a separate cluster and share a close phylogenetic relation with each other. Unlike the Acanthopterygii VT and IT precursors, the Ostariophysi VT and IT precursors are phylogenetically distant and share an evolutionary distance of 0.422 (Figure 5).

Linkage and Synteny Analysis of VT and IT in Teleosts and Spotted Gar

Details of the chromosome blocks and linkage groups used in the analysis are summarized in Supplementary Table 2. A schematic representation of conserved synteny in the spotted gar and teleosts is presented in Figure 6. In the spotted gar, VT, IT and the novel peptide precursors are present in the same linkage group. But, the VT precursor gene (*avp*-like) and IT precursor genes (*oxl*-like) are not arranged in tandem, unlike in human and *Xenopus* where the nonapeptide genes are present in tandem. However, the *oxl*-like gene and the novel peptide precursor gene (both in the neutral family) are arranged in tandem. Similarly, in acanthopterygian teleosts (fugu, tilapia, medaka, stickleback), the *avp*-like (VP-like) and *oxl*-like genes are in the same linkage group but are not present in tandem.

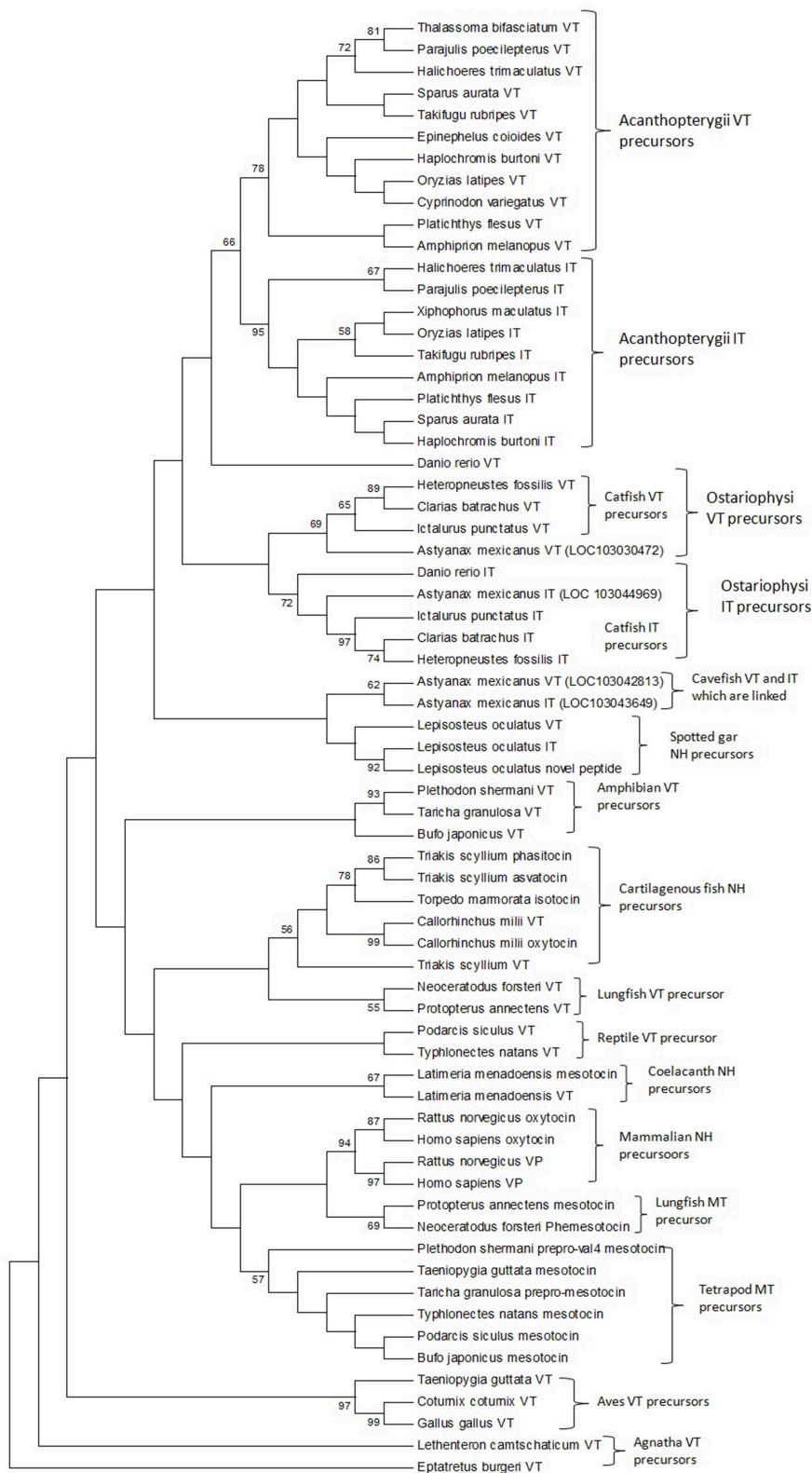


FIGURE 4 | The phylogenetic tree of vertebrate neurohypophyseal nonapeptides constructed by the Maximum-Likelihood method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. The analysis involved 67 amino

acid sequences. All positions with less than 95% site coverage were eliminated. There were a total of 97 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. References to the sequence and species name are given in Supplementary Table 1.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1. Ostariophysi IT | | 0.055 | 0.060 | 0.071 | 0.061 | 0.064 | 0.054 | 0.067 | 0.064 | 0.066 | 0.076 | 0.065 | 0.075 | 0.065 | 0.060 | 0.060 | 0.061 | 0.068 | 0.069 | 0.081 | 0.050 |
| 2. Acanthopterygii IT | 0.395 | | 0.045 | 0.070 | 0.070 | 0.068 | 0.060 | 0.076 | 0.069 | 0.077 | 0.084 | 0.071 | 0.076 | 0.068 | 0.075 | 0.066 | 0.072 | 0.079 | 0.080 | 0.090 | 0.058 |
| 3. Acanthopterygii VT | 0.452 | 0.269 | | 0.075 | 0.069 | 0.070 | 0.054 | 0.072 | 0.066 | 0.071 | 0.073 | 0.062 | 0.070 | 0.062 | 0.070 | 0.061 | 0.069 | 0.074 | 0.072 | 0.084 | 0.056 |
| 4. Cartilaginous fish neutral hormone | 0.561 | 0.511 | 0.530 | | 0.062 | 0.066 | 0.078 | 0.051 | 0.059 | 0.068 | 0.081 | 0.069 | 0.068 | 0.071 | 0.071 | 0.061 | 0.060 | 0.077 | 0.080 | 0.093 | 0.067 |
| 5. Coelacanth MT | 0.424 | 0.416 | 0.461 | 0.429 | | 0.049 | 0.064 | 0.060 | 0.047 | 0.039 | 0.078 | 0.056 | 0.055 | 0.063 | 0.060 | 0.043 | 0.043 | 0.070 | 0.073 | 0.082 | 0.055 |
| 6. Lungfish MT | 0.463 | 0.440 | 0.482 | 0.485 | 0.258 | | 0.067 | 0.064 | 0.061 | 0.060 | 0.071 | 0.070 | 0.059 | 0.062 | 0.061 | 0.049 | 0.047 | 0.058 | 0.062 | 0.088 | 0.064 |
| 7. Ostariophysi VT | 0.422 | 0.426 | 0.384 | 0.593 | 0.437 | 0.476 | | 0.070 | 0.064 | 0.065 | 0.074 | 0.064 | 0.071 | 0.066 | 0.070 | 0.059 | 0.068 | 0.067 | 0.068 | 0.078 | 0.058 |
| 8. Cartilaginous fish VT | 0.517 | 0.530 | 0.489 | 0.350 | 0.386 | 0.440 | 0.524 | | 0.054 | 0.067 | 0.077 | 0.071 | 0.070 | 0.061 | 0.072 | 0.051 | 0.060 | 0.076 | 0.078 | 0.093 | 0.065 |
| 9. Lungfish VT | 0.476 | 0.440 | 0.410 | 0.409 | 0.257 | 0.398 | 0.469 | 0.343 | | 0.052 | 0.075 | 0.052 | 0.062 | 0.058 | 0.064 | 0.043 | 0.055 | 0.072 | 0.072 | 0.083 | 0.055 |
| 10. Coelacanth VT | 0.455 | 0.500 | 0.458 | 0.466 | 0.132 | 0.349 | 0.440 | 0.417 | 0.277 | | 0.078 | 0.058 | 0.072 | 0.063 | 0.071 | 0.048 | 0.057 | 0.070 | 0.073 | 0.081 | 0.063 |
| 11. Agnatha VT | 0.608 | 0.628 | 0.564 | 0.656 | 0.580 | 0.546 | 0.581 | 0.595 | 0.589 | 0.571 | | 0.079 | 0.086 | 0.073 | 0.075 | 0.072 | 0.081 | 0.084 | 0.081 | 0.096 | 0.073 |
| 12. Reptile VT | 0.453 | 0.445 | 0.399 | 0.481 | 0.244 | 0.408 | 0.434 | 0.457 | 0.264 | 0.257 | 0.590 | | 0.063 | 0.066 | 0.070 | 0.047 | 0.058 | 0.070 | 0.069 | 0.084 | 0.061 |
| 13. Reptile MT | 0.559 | 0.486 | 0.478 | 0.516 | 0.298 | 0.363 | 0.520 | 0.490 | 0.408 | 0.416 | 0.668 | 0.341 | | 0.072 | 0.060 | 0.054 | 0.042 | 0.069 | 0.075 | 0.109 | 0.066 |
| 14. Avian VT | 0.471 | 0.447 | 0.403 | 0.522 | 0.386 | 0.400 | 0.465 | 0.440 | 0.371 | 0.385 | 0.565 | 0.401 | 0.487 | | 0.072 | 0.057 | 0.066 | 0.066 | 0.066 | 0.091 | 0.063 |
| 15. Avian MT | 0.438 | 0.481 | 0.459 | 0.520 | 0.326 | 0.341 | 0.501 | 0.483 | 0.401 | 0.416 | 0.571 | 0.432 | 0.298 | 0.475 | | 0.056 | 0.045 | 0.058 | 0.069 | 0.097 | 0.059 |
| 16. Amphibian VT | 0.469 | 0.463 | 0.458 | 0.468 | 0.271 | 0.347 | 0.450 | 0.372 | 0.292 | 0.299 | 0.573 | 0.304 | 0.369 | 0.403 | 0.377 | | 0.045 | 0.060 | 0.064 | 0.076 | 0.054 |
| 17. Amphibian MT | 0.475 | 0.467 | 0.495 | 0.436 | 0.235 | 0.296 | 0.507 | 0.422 | 0.360 | 0.331 | 0.625 | 0.351 | 0.224 | 0.467 | 0.249 | 0.322 | | 0.058 | 0.064 | 0.092 | 0.060 |
| 18. Mammalian OT | 0.478 | 0.513 | 0.499 | 0.558 | 0.419 | 0.328 | 0.485 | 0.523 | 0.476 | 0.411 | 0.624 | 0.427 | 0.411 | 0.411 | 0.307 | 0.424 | 0.368 | | 0.046 | 0.096 | 0.070 |
| 19. Mammalian VP | 0.480 | 0.513 | 0.473 | 0.561 | 0.440 | 0.345 | 0.477 | 0.527 | 0.428 | 0.440 | 0.616 | 0.370 | 0.472 | 0.390 | 0.400 | 0.438 | 0.414 | 0.219 | | 0.089 | 0.074 |
| 20. Spotted gar VT | 0.560 | 0.549 | 0.549 | 0.667 | 0.503 | 0.594 | 0.534 | 0.615 | 0.513 | 0.484 | 0.730 | 0.503 | 0.741 | 0.563 | 0.626 | 0.532 | 0.604 | 0.620 | 0.543 | | 0.075 |
| 21. Spotted gar neutral peptide precursor | 0.341 | 0.333 | 0.354 | 0.480 | 0.334 | 0.416 | 0.412 | 0.444 | 0.342 | 0.395 | 0.567 | 0.357 | 0.436 | 0.395 | 0.373 | 0.394 | 0.411 | 0.434 | 0.451 | 0.415 | |

FIGURE 5 | Shows the number of amino acid substitutions per site from averaging overall sequence pairs between groups. Standard error estimate(s) shown above the diagonal were obtained by a bootstrap procedure (500 replicates). Analyses were conducted using the Poisson correction model (Zuckerkandl and Pauling, 1965). The analysis involved 67

amino acid sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 97 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

In the ostariophysian teleosts, two contrasting situations exist. In zebrafish, *avp*-like and *oxt*-like are present on two different chromosomes. In contrast, in the cavefish where there are two genes each for VT and IT, one pair is present in the same linkage group like in the acanthopterygian fish. The other *avp*-like and *oxt*-like genes are present on different linkage groups so that in the cavefish, there are three linkage groups harboring the nonapeptide genes. The chromosome block in which the two genes are linked shares a conserved synteny with the acanthopterygian NH loci and zebrafish *avp*-like containing linkage group. The other *oxt*-like gene is present in the paralogon of this block and shares a conserved synteny with the zebrafish *oxt*-like containing block and the Acanthopterygii paralogon of the NH loci. The genomic context of the other *avp*-like gene is considerably different from that of the teleost and spotted gar nonapeptide-harboring chromosome blocks or their paralogons. But, on a closer examination it seems to share a conserved synteny with the tetrapod nonapeptide loci as it is present in the same linkage group as the genes *fast kd 5* and *ddrgk* (genes linked with NH genes in tetrapods) but separated from them by 13 genes.

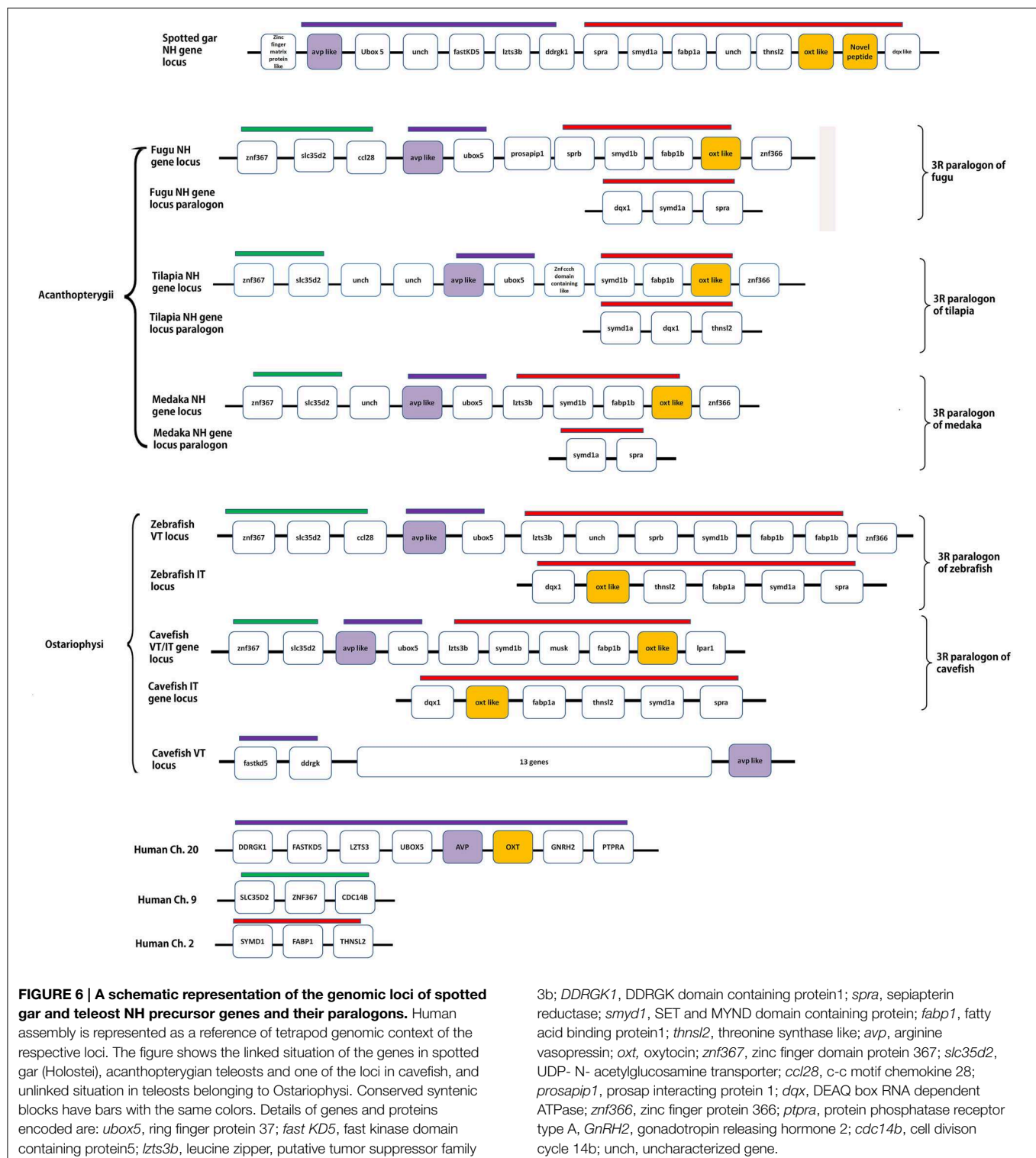
Discussion

Catfish VT and IT Precursor Genes and Proteins

In the present study, we cloned a full length cDNA of 618 bp from *H. fossilis* that coded for a VT precursor of 155 aa. There is no difference in the sequence of the brain and ovarian transcripts and hence the same gene expresses in both the tissues. We could also clone *C. batrachus* partial VT precursor gene from the N-terminal neurophysin coding region to the 3' UTR. The catfish VT precursor gene does not show any major departure from the other known teleost sequences. However, a repeat of CA dinucleotide at the 3' UTR is unique and the number of repeats is varied in different catfishes. In *H. fossilis*, there are 19 repeats,

while in *C. batrachus*, there are only 4 repeats. The putative *Ictalurus* VT precursor gene from the database do not show any CA repeat. The available sequence read ended with the coding region of the neurophysin and did not extend to the 3' UTR region so that no CA repeat was observed. It may be inferred that the clone has CA repeats in the 3' UTR, as it belongs to the microsatellite enriched library. It is very likely that the CA repeats may be a conserved feature of the VT precursor genes of catfishes and may have a regulatory role.

The deduced amino acid sequence of the VT precursors shows that, like all other members of neurohypophyseal nonapeptide precursor superfamily, it has a multi-domain structure, each having a separate function. Apart from the signal peptide, the vasopressin family peptides have a three domain structure. The nine amino acid hormone forms the smallest domain, the most biologically active region, and is released with a C-terminal amidation after a three-step enzymatic process. The second domain called neurophysin is joined to the hormone moiety with a GKR, which is the site of the three-step enzymatic process of cleavage of the hormone and amidation. The N-terminal 9 amino acid of the neurophysin is poorly conserved, while the rest is conserved. The third domain is copeptin which is poorly conserved except for the presence of a leucine-rich core (de Bree and Burbach, 1998). The catfish VT precursors like the vasopressin family peptides have the three domain structure. Only in mammals, a second cleavage apart from the hormone-neurophysin cleavage has been reported, which separates the copeptin from the neurophysin. The copeptin domain possesses N-linked glycosylation sites in mammals, amphibians and lungfish (Hyodo et al., 1997) but unlike in teleosts. The catfish VT copeptin domain does not have a N-linked glycosylation site nor is cleaved. The isolated IT precursor has a multi-domain structure too and, like VT/VP precursors, has an extended C-terminal with a leucine-rich core, while no other vertebrate prohormone in the neutral line has got an extended C-terminal.



Tissue Expression Profile of Nonapeptides and Functional Implications

In *H. fossilis*, VT and IT genes are expressed only in the brain and ovary. In a previous study, the distribution of VT has been reported in the preoptico-hypophyseal neurosecretory system of the catfish (Singh and Joy, 2008). Brain VT showed seasonal

variation associated with the annual reproductive cycle of the catfish with an increase during the recrudescence phase and a decrease after spawning. The expression of the VT and IT genes shows differences in tissue and seasonal transcript levels. The transcript levels are high in the brain than ovary. In the ovary, the expression was confined to the follicular layer since the denuded

oocytes did not show any signal. In the seasonal study, the VT and IT precursor transcripts in the brain and ovary show significant variations with higher levels during the reproductive phase, suggesting a prominent influence of the reproductive factors on the transcriptional activity of the genes. The expression of both VT and IT precursor genes in the ovary points to local synthesis of the peptides for a paracrine role in ovarian activity. This confirms the earlier findings from our laboratory showing HPLC characterization of both VT and IT, and immunocytochemical localization of VT in the follicular layer (Singh and Joy, 2008). The ovarian VT levels vary with the annual ovarian cycle closely associated with recrudescence and spawning (Singh and Joy, 2008). Moreover, steroid hormones estradiol-17 β and 17, 20 β - dihydroxypregnen-3-one, the maturation- inducing steroid, modulate VT secretion as in the brain (Singh and Joy, 2009a, 2011). *In vitro* studies with ovarian follicles have showed that VT stimulated steroidogenesis, oocyte final maturation, oocyte hydration, ovulation and prostaglandin secretion (Singh and Joy, 2010, 2011; Joy and Singh, 2013). Gwee et al. (2009) have shown VT expression in the ovary of elephant shark. Bobe et al. (2006) have reported VT and IT mRNAs in the preovulatory ovaries of rainbow trout, while the transcripts were not detected in the ovary during the other stages of reproduction. In mammals, there are several reports of the presence of VP and OT in nonneuronal peripheral sites, where they have a paracrine role (Wathes et al., 1983; Clements and Funder, 1986; Sernia et al., 1994; Mechaly et al., 1999). Similarly, in other classes of vertebrates like aves, presence of VT has been reported in the reproductive tissues (Saito and Grossmann, 1999). The peripheral expression of VT and its homolog signifies a phylogenetic pattern and the phenomenon seems to be conserved in all major groups of vertebrates.

Evolutionary History of Teleost NH Precursors from Phylogenetic Analysis, Genomic Locations and Synteny Analysis

Phylogenetic inference from the present study shows that the precursor paralogs in some vertebrate groups (cartilaginous fish, coelacanth, mammals) are closer than their homologs in other groups. Similarly, studies in salmon (Hyodo et al., 1991; Suzuki et al., 1992) and flounder (Warne et al., 2000) showed that the VT and IT precursors share high sequence identity. Gwee et al. (2008, 2009) also showed that the sequence identity between the respective precursor paralogs is very high in coelacanth and elephant shark. The studies also reported that the high sequence similarity was mostly in the central portion of the neurophysin. Events of gene conversion between the paralogous genes may be responsible for diluting the distance created by the long evolutionary history and make them seem more closely related than what they actually are, typifying “concerted evolution.” Concerted evolution is common in duplicated genes, for example, as reported in rRNA genes, the highly conserved histone and ubiquitin gene families, and the heat shock protein gene family (Nei and Rooney, 2005). For the NH precursors, gene conversions seem to encompass the central neurophysin region and, therefore, the high sequence similarity in the central portion. Further evidence in favor of frequent events of gene conversion

in between the nonapeptide paralogs is given for the bovine VP and OT precursor mRNA and avian NH precursors, having their central neurophysin not merely similar but identical pointing to a recent event of gene conversion (Ruppert et al., 1984; Levy et al., 1987). Gwee et al. (2008) found a low GC3 content in all the three exons of coelacanth VT and MT precursors as compared to bovine and human VP and OT genes, and attributed the sequence similarity in coelacanth to purifying selection rather than due to gene conversion. In mammals and yeast, gene conversion events are known to be GC-biased so that the GC content increases after gene conversion (Galtier, 2003; Marais, 2003; Noonan et al., 2004; Duret and Galtier, 2009). However, a recent study showed that gene conversions might not always be GC-biased (Assis and Kondrashov, 2012) and in such a scenario one of the evidence in favor of gene conversion can be incompatibility between phylogenetic tree and known duplication history (Mansai and Innan, 2010), as is the case in the present study. The neurophysin is only concerned with precursor processing and axonal transport which is common to all nonapeptides in contrast to the hormone moieties that evolved under the constraints of stringent and differential ligand-receptor selectivity in the two families. Hence, gene conversion events encompassing only the neurophysin might have occurred during evolution. This might also be facilitated by the 3-exon structure of the nonapeptide genes where the central neurophysin is encoded by a separate exon, i.e., the 2nd exon.

Based on the gene structures of salmon VT and IT, Urano and Ando (2011) had proposed a distinct and independent origin of the teleost IT as compared to the neutral hormone precursors of other vertebrates. Evidence in support of it is the presence of an extended C-terminal in IT precursors with a leucine-rich core, like in VT/VP. However, the authors have added that the molecular divergence of VT and IT in teleosts is complicated because of the 3R. The recent addition of the spotted gar sequences in the database and the analysis in the present study has prompted a re-appraisal. The presence of IT with the extended C-terminal in the spotted gar suggests an origin of IT in the actinopterygian lineage before the 3R. The spotted gar has an additional neutral peptide with a short C-terminal, which may be related directly to the evolutionary line of other vertebrate neutral hormone precursors that has originated at the base of the gnathostome lineage. In no teleost studied till date, a neutral peptide with a short C-terminal has been reported. Therefore, it may be hypothesized that the neutral hormone precursor gene with the short C-terminal might have originated very early in vertebrates, as documented in the cartilaginous fish (Hyodo et al., 2004; Gwee et al., 2009) and this early neutral hormone precursor might be the evolutionary forerunner of MT and OT. In teleosts, this neutral hormone precursor gene might have been secondarily lost or needs to be traced in the teleost lineage.

An interesting result that surfaced due to the addition of the catfish nonapeptide precursor sequences in the phylogenetic study is about the differences in the evolutionary distance shared by the precursor paralogs of different groups within teleosts. While the Acanthopterygii VT and IT precursors share a very small evolutionary distance (0.269) between them, the Ostariophysi VT and IT precursors are phylogenetically

distant (0.422). The distance between the VT precursors and IT precursors of Acanthopterygii and Ostariophysi is also high (0.426 and 0.452, respectively). Phylogenetic clustering of the teleost NH precursors further supports these results. In the phylogenetic tree, the Acanthopterygii VT and IT precursors clustered together and seemed to have diverged from a common lineage. While the Ostariophysi IT precursors formed a definite cluster that included the catfish IT precursors, one of the IT precursors of the cavefish and the zebrafish IT precursor. The VT precursors of the catfish and one of the cavefish make a cluster which does not include the zebrafish VT precursor. The results from the distance calculation between the groups and the phylogenetic tree suggest that underlying the apparent homogeneity in all teleosts having VT and IT, there might be a difference in the origin of VT and IT precursors in different groups, i.e., these may be lineage-specific paralogs.

The analysis of the genomic loci of the NH hormone genes and the synteny analysis of the loci in different groups of teleosts further support the above assumption. Gwee et al. (2008, 2009) showed that in coelacanth, lamprey, and elephant shark (cartilaginous fish), the NH gene loci has a conserved synteny with the tetrapod NH loci. In *fugu*, there is a rearrangement. In the present study, the NH gene loci were studied in the spotted gar and teleosts belonging to different groups, which are known to have suffered independent gene losses during the process of diploidization (Garcia de la serrana et al., 2014). Our study indicates that, indeed, the loci and 3R paralogs show the footprints of rearrangements and differential losses of the NH gene paralogs in the different teleost lineages. On examination of the different genes linked with the nonapeptide genes in the spotted gar chromosome segment and the human linkage groups (as a tetrapod reference), it may be inferred that a rearrangement in the position of the genes in the nonapeptide loci of the spotted gar occurred by the fusion of the two blocks of chromosomes after the origin of IT in the actinopterygian lineage, disrupting the tandem arrangement of the genes, before the 3R. After the 3R, in teleosts the two paralogous blocks (3R paralogs) harboring the nonapeptide genes suffered further rearrangement and differential gene losses during the process of diploidization. The rearrangement was common judging from the conserved synteny in both the NH gene loci and its paralogon in all teleosts. However, the gene losses in these loci were differential in the two teleost superorders Acanthopterygii and Ostariophysi. This led to the linked and nonlinked *avp*-like and *oxl*-like genes in these different groups. In the acanthopterygian lineage, both the genes might have been lost from the same paralogon maintaining the linked situation of the two genes similar to the spotted gar, i.e., the pre 3R genome. In the zebrafish, *avp* like and *oxl*-like genes were lost one each from two paralogs so that a single copy of the NH hormone genes was maintained and they remained unlinked. It is difficult to reconstruct the sequence of events that can explain the present picture of synteny in the NH loci and also the inconsistent phylogenetic relation between the VT and IT precursors in the different groups of teleosts. However, it may be inferred that in the base lineage of acanthopterygians, after one pair of VT and IT precursor genes was lost from one of the 3R paralogon, an event of gene conversion might have occurred

between the remaining pair. This might have occurred in the time period between the 3R and the explosive adaptive radiation and could explain the phylogenetic relation of the Acanthopterygii VT and IT precursors. The Acanthopterygii VT precursors and Acanthopterygii IT precursors make two distinct clusters but share a very close phylogenetic relation. In the Ostariophysi, the loss of the NH precursor paralog was differential as compared to the Acanthopterygii with VT and IT precursor genes being lost one each from the two 3R paralogs. Rapid speciation event might have occurred after this. Gene conversion might not have occurred in the ostariophysian lineage. This explains the unlinked state of the NH paralogs in the zebrafish and also the distant relation shared by the VT and IT precursors of the Ostariophysi. The situation in the cavefish is difficult to explain. It is likely that the VT precursor gene was lost from one of the 3R paralogs while in the other both genes were retained so that a pair of linked genes remained. An independent event of gene conversion might also have occurred in between the two linked genes explaining the close phylogenetic relation between the two. The evolutionary history of the VT precursor gene of the cavefish which is unlinked can be easily traced phylogenetically as it clusters with the catfish VT precursors. However, the synteny analysis shows that it is present in a unique genomic context which shares a conserved synteny with neither the tetrapod nor the teleost NH loci, making it impossible to arrive at a possible origin of this gene. Genomic information of the NH loci from more teleost species belonging to the Ostariophysi may be helpful in explaining this. Our future direction of work would involve isolating the NH gene loci in the catfish so that the evolutionary history of the VT and IT genes in the Ostariophysi may be inferred more clearly. With the available information from the phylogeny and synteny analysis, it is clear that in the different groups of teleosts the VT and IT precursors are lineage-specific paralogs arising from differential loss of the 3R paralogs in the different superorders. The independent yet consistent retention of VT and IT in the different groups despite the catastrophes of genome duplications, diploidizations and gene conversions might have been directed by stringent ligand- receptor selectivity, established early during the vertebrate evolution.

Conclusion

The complete VT and IT precursors were cloned from *H. fossilis*, and partially from *C. batrachus*. The deduced precursor proteins have the characteristics of the NH precursors. The precursors express in the brain and follicular envelope of the ovary. The expression in both brain and ovary shows seasonal variation with higher expression in the reproductive phases. The phylogenetic analysis of the vertebrate NH precursors and the synteny analysis of the NH gene loci show that the VT and IT precursors are lineage-specific paralogs in the superorders of Teleostei (Acanthopterygii and Ostariophysi).

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Supplementary Material

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AMPK: a master energy regulator for gonadal function

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From *C. elegans* to mammals (including humans), nutrition and energy metabolism significantly influence reproduction. At the cellular level, some detectors of energy status indicate whether energy reserves are abundant (obesity), or poor (diet restriction). One of these detectors is AMPK (5' AMP-activated protein kinase), a protein kinase activated by ATP deficiency but also by several natural substances such as polyphenols or synthetic molecules like metformin, used in the treatment of insulin resistance. AMPK is expressed in muscle and liver, but also in the ovary and testis. This review focuses on the main effects of AMPK identified in gonadal cells. We describe the role of AMPK in gonadal steroidogenesis, in proliferation and survival of somatic gonadal cells and in the maturation of oocytes or spermatozoa. We discuss also the role of AMPK in germ and somatic cell interactions within the cumulus-oocyte complex and in the blood testis barrier. Finally, the interface in the gonad between AMPK and modification of metabolism is reported and discussion about the role of AMPK on fertility, in regards to the treatment of infertility associated with insulin resistance (male obesity, polycystic ovary syndrome).

Keywords: AMPK, testis, ovary, germ cells, fertility

Introduction

The 5' AMP-activated protein kinase (AMPK) is a serine/threonine heterotrimeric kinase composed of one catalytic α -subunit bound with β - and γ -regulatory subunits. The genes encoding the three subunits of AMPK are highly conserved in eukaryotic species including vertebrates, invertebrates, plants, fungi, and protozoa (Hardie et al., 2003; Ghillebert et al., 2011). Activation of AMPK occurs with the phosphorylation of the α -subunit at Threonine 172. AMPK is sensitive to the AMP to ATP ratio and is activated by an increasing AMP concentration and by the upstream kinases including liver kinase B1 (LKB1) and calcium/calmodulin (CaM) kinase (CaMKK) (Woods et al., 2003; Hawley et al., 2005). It can also be dephosphorylated by phosphatases [Protein kinase phosphatase-1 and -2A (PP2A and PP2C)] (Lu et al., 2010; Joseph et al., 2015). AMPK is activated in pathophysiological situations (exercise, stress), by metabolic hormones (leptin, adiponectin, ghrelin) or pharmacological agents [5-aminoimidazole-4-carboxamide-1- β -D-ribose (AICAR), metformin and thiazolidinediones (TZD)] (Hardie, 2015). It regulates energy homeostasis by maintaining constant intracellular ATP concentrations by stimulation of catabolic pathways and inhibition of anabolic pathways (Hardie, 2015). Several studies have also shown that AMPK is expressed in gonads (Tosca et al., 2005; Dupont et al., 2012; Tartarin et al., 2012a) and could play a key role in the reproductive function in linking the gonadal axis with energy balance. More precisely, AMPK is present in ovarian (granulosa and theca cells, oocytes and corpora lutea) and testicular (Sertoli, Leydig and germinal cells) cells in different species [oyster (Guévelou et al., 2013),

C. elegans (Lee et al., 2008), bird (Tosca et al., 2006a; Nguyen et al., 2014), mammals: cow (Tosca et al., 2007a), pig (Mayes et al., 2007), rodents (Tosca et al., 2005; Downs et al., 2010; Tartarin et al., 2012a) and human (Pellatt et al., 2011)]. This kinase controls gonad steroidogenesis and germinal cell maturation but also cell proliferation and survival, polarity, formation, and maintenance of cellular junctional complexes, and cytoskeletal dynamics. In this review we report briefly some of the known functions of AMPK in the female and male gonad, and then we describe the potential role of this kinase in the interactions between metabolism and gonadal function. Most of the studies and conclusions are based on animal studies. However, we reported human studies (about granulosa and thecal cells or human embryonic testis) when it was possible.

Gonadal Steroidogenesis

In female mammals and birds, the role of AMPK has been studied in detail in granulosa cell cultures by using pharmacological agents and adenovirus-mediated expression of dominant negative forms of AMPK (Tosca et al., 2005). AMPK activators inhibit the secretion of progesterone and/or estradiol by granulosa cells in mammals (Tosca et al., 2005, 2007a). In rat and bovine species, this inhibition is associated with a decrease in 3 β -hydroxysteroid dehydrogenase (3 β -HSD) mRNA and protein levels and a decrease in MAP kinase (MAPK) extracellular signal-regulated kinases (ERK) 1/2 phosphorylation (Tosca et al., 2005, 2007a, 2010). In rats, AMPK activation induced by metformin does not reduce aromatase expression and estradiol production. However, it decreases progesterone synthesis and the expression of different proteins involved in steroidogenesis [3 β -HSD, cytochrome P450 (CYP11A1), steroidogenic acute regulatory protein (StAR)] (Tosca et al., 2006b). Metformin reduces follicle-stimulating hormone (FSH) but not forskolin-stimulated aromatase expression and activity in an AMP-activated protein kinase-independent manner in a human granulosa cell line (Rice et al., 2013). Also in human granulosa cells, metformin decreases androgen synthesis, by directly inhibiting cytochrome P450 17 α -hydroxylase (Cyp17) activity (La Marca et al., 2000). In contrast, it has been shown that AMPK could improve androgen production by adrenal cells (Hirsch et al., 2012). Even if no studies have shown a role of AMPK in ovarian steroidogenesis *in vivo*, this has been largely demonstrated *in vitro*.

A total α 1AMPK knock out mouse model has been developed (Tartarin et al., 2012a). The male α 1AMPK^{-/-} have high levels of testosterone that are not due to adrenal disorders or to glucocorticoid resistance but to hyperactive Leydig cells (Tartarin et al., 2012a). Indeed, the Leydig cells of these animals have an increased volume, an altered endoplasmic reticulum area, a high intratesticular cholesterol concentration and a greater expression of proteins involved in steroid production (Tartarin et al., 2012a). These data accord with those described previously *in vitro* in granulosa cells but also in Leydig cells in response to a modulation of AMPK activity either pharmacologically or genetically. Certainly in MA-10 and MLTC-1 Leydig cells, AMPK activation inhibits cAMP-induced steroidogenesis by repressing

the expression of key regulators of steroidogenesis, including the cholesterol carrier, StAR and the nuclear receptor Nr4a1 (Abdou et al., 2014). In the latter study, the authors suggest that some AMPK-sensitive element(s) containing sites for nuclear receptors of NR4A1 are located in the StAR promoter and are required for elevated cAMP dependent activation (Abdou et al., 2014). This suggests that activation of AMPK reduce the activity of NR4A1 and StAR expression. Furthermore, in primary rat Leydig cells, resveratrol, an AMPK agonist impairs human chorionic gonadotropin (hCG)-mediated testosterone production by repressing StAR expression (Svechnikov et al., 2009). In humans, the association of increased steroid production and the inhibition of AMPK could be associated to the Peutz-Jeghers Syndrome (PJS) (Ham et al., 2013). Peutz-Jeghers syndrome is an autosomal-dominant disorder that arises as a consequence of mutations in the serine/threonine kinase 11 (STK11) gene that encodes LKB1. In these PJS patients, excess estrogen and an increase in testicular aromatase expression is associated with a decrease in AMPK phosphorylation in the testis (Ham et al., 2013). Thus, AMPK could be a molecular modulator that inhibits gonadal steroidogenesis to preserve cellular energy homeostasis and prevent excess steroid production.

An important study for human health found that when human and mouse fetal testes were cultured in the presence of metformin, there was a reduction in testosterone secretion and mRNA of key factors which are involved in steroidogenesis (Tartarin et al., 2012b). This was also associated with an increase in lactate production. Furthermore, *in vivo* administration of metformin during pregnancy reduced the testicular size of fetal and neonatal testes. Although the number of germ cells was not altered by metformin treatment, the number of Sertoli cells was reduced in both fetal and neonatal testes. Interestingly the androgen producing Leydig cell population was only reduced in the fetal period at 16 days post-coitum (Tartarin et al., 2012b). This study presented a potentially harmful effect of metformin treatment on the development of fetal testes (Tartarin et al., 2012b). These effects were likely to result from a metformin-stimulated AMPK-mediated reduction in cellular proliferation (Kayampilly and Menon, 2012), indicating that the reduction in steroidogenesis occurred as a result of reduced testicular growth.

Proliferation and Survival of Somatic Gonadal Cells

Gonadal somatic cells comprise the granulosa, cumulus and theca cells of the ovary, and the Sertoli and Leydig cells of the testis. Proliferation and survival of somatic cells are indispensable for fertility. Indeed, it is well known that proliferating granulosa cells support the progression of follicular growth and oocyte maturation. In males, testis size and sperm production are directly correlated to the total number of adult Sertoli cells. Regulation of proliferation and survival processes involves different hormones including FSH. As AMPK has previously been described as inhibiting proliferation of somatic cells (Tosca et al., 2010; Hardie, 2011; Kayampilly and Menon, 2012; Riera

et al., 2012), we will examine the proliferative role of AMPK for these critical cell types.

In *C. elegans*, AMPK promotes survival and arrests germline development during nutrient stress (Fukuyama et al., 2012). More precisely, AMPK α 1 and AMPK α 2 (aak-1 and aak-2), the two catalytic α subunits of AMP-activated protein kinase, regulate germline quiescence by suppressing activity of target of rapamycin complex 1 (TORC1) that is involved in cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription. Similarly in rat Sertoli cells, Riera et al. (2012) observed that activation of AMPK induces a decrease in FSH-stimulated Sertoli cell proliferation through a phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mTORC1 mechanisms but also an increase in cyclin dependent kinase inhibitor (CDKI, p19INK4d, p21Cip1, and p27Kip1) expression (Riera et al., 2012). In agreement with these data, Tosca et al. (2010) observed that metformin-induced AMPK activation reduces cell growth, protein synthesis and MAPK ERK1/2 and ribosomal protein S6 kinase (p90rsk) phosphorylation in response to insulin-like growth factor 1 (IGF1) in cultured bovine granulosa cells. Furthermore, Kayampilly and Menon observed that exposure of rat granulosa cells with a pharmacological activator of AMPK increased p27kip expression, an inhibitor of the cell cycle (Kayampilly and Menon, 2009). These latter authors have also observed that activation of AMPK induced by dihydrotestosterone (DHT) treatment decreases granulosa cell mitogenesis and consequently could contribute to ovulatory dysfunction observed in hyperandrogenic states (Kayampilly and Menon, 2012).

Maturation of Germ Cells

The Oocyte

The role of AMPK in mammalian oocyte maturation is strikingly species specific. AMPK improves resumption of oocyte meiosis in mice (Chen et al., 2006; Downs and Chen, 2006; Larosa and Downs, 2007; Chen and Downs, 2008) but not in rats (Downs, 2011) and pharmacological activation of AMPK blocks nuclear oocyte maturation in pigs and cattle (Mayes et al., 2007; Tosca et al., 2007b; Santiquet et al., 2014). The oocyte is reliant on the metabolism of lactate and pyruvate from the tricarboxylic acid cycle (TCA cycle) and oxidative phosphorylation for most of its energy stores (Biggers et al., 1967; Leese and Barton, 1984; Roberts et al., 2002). In human granulosa cells, AMPK could be also involved in lactate production which is important for follicular development (Richardson et al., 2009). The cumulus-oocyte complex (COC) also metabolizes glucose via numerous important pathways such as the pentose phosphate pathway and glycolysis. These metabolic pathways are critical for successful oocyte maturation and resumption of meiosis (Downs and Mastropolo, 1994; Downs et al., 1996; Sutton et al., 2003a,b). Cyclic adenosine monophosphate (cAMP), synthesized by adenylate cyclase downstream of the pentose phosphate pathway, is an important negative regulator of meiotic maturation. It is well known that degradation of cAMP by phosphodiesterases triggers resumption of meiosis. The depletion of cAMP results in an increase in the AMP/ATP ratio. As AMP

levels rise, AMPK is triggered and activates a number of enzymes involved in energy producing pathways and inhibiting energy consuming pathways (Downs et al., 2002). This makes AMPK critically important for oocyte developmental competence.

In mouse oocytes, the α 1AMPK subunit is more abundant than α 2AMPK. Immunolocalization of the α 1 catalytic subunit of AMPK showed an association with condensed chromatin and the meiotic spindle but not in the spindle poles or midbody. In the absence of α 1AMPK specifically in the oocyte, a decrease in mdm2 protein level, a strong negative regulator of p53, leads to an increase in the p53 content and probably induces cell cycle arrest as shown by the few number of oocyte fertilized in IVF or by the lower litter size in comparison to control mice (Bertoldo et al., 2014b, 2015). AMPK activation increases the rate of germinal vesicle breakdown (GVBD), spindle formation and polar body (PB) extrusion whereas the kinase has no effect on peripheral movement of the spindle. The meiosis-inducing actions and localization of AMPK are regulated by microtubule spindle integrity during mouse oocyte maturation (Ya and Downs, 2014). Interestingly, in mice, fatty acid oxidation is required for AMPK-induced maturation *in vitro* (Vasankar and Downs, 2013).

The AMPK activator, AICAR, is a potent stimulator of maturation in mouse cumulus cell-enclosed oocytes (CEO) and denuded oocytes (DO), but only marginally stimulatory in rat CEO and ineffective in rat DO (Downs, 2011). AICAR and compound C produced contrasting results on polar body formation in cultured CEO in rat and mouse. Active AMPK was colocalized with chromatin after GVBD in rat and mouse oocytes, but did not appear at the spindle poles in rat oocytes as it did in mouse oocytes. These data highlight significant differences in meiotic regulation between the two species (Downs, 2011).

Contrary to results obtained with mouse oocytes, bovine and porcine oocyte meiosis is inhibited by activators of AMPK which is activated by AMP, the degradation product of cAMP (Bilodeau-Goeseels et al., 2007; Mayes et al., 2007; Bilodeau-Goeseels, 2011). During oocyte *in vitro* maturation (IVM), Santiquet et al. (2014) cultured porcine oocytes in the presence of AICAR and assessed the oocytes response in reference to oocyte nuclear maturation and cumulus cell expansion. Nuclear maturation was inhibited, however, this effect was only observed in cumulus enclosed oocytes, suggesting that cumulus cells are essential for AICAR's effect on oocyte maturation. In addition, AICAR inhibited cumulus expansion, which normally occurs in response to FSH and/or epidermal growth factor (EGF) during IVM (Harper and Brackett, 1993; Lonergan et al., 1996; Ritter et al., 2015). The results in porcine are supported by those in bovine where supplementation of metformin during embryo *in vitro* production resulted in AMPK mediated activation of TSC2 (Pikiou et al., 2015) and probably a reduction in TOR complex signaling and protein synthesis inhibition. In bovine COCs, metformin blocks meiotic progression at the germinal vesicle stage, activates AMPK, and inhibits MAPK3/1 phosphorylation in both the oocytes and cumulus cells during *in vitro* maturation. Moreover, cumulus cells were essential for the effects of metformin on bovine oocyte maturation, whereas MAPK ERK1/2 phosphorylation was not (Tosca et al., 2007b). While the precise targets of AMPK in the COCs are not entirely

known, AMPK has been shown to modulate protein synthesis in various cell types (Hormon et al., 2002; Proud, 2004) and proteins involved in communication with somatic cells (see next section).

As observed in the large animal species, AMPK signaling keeps nemertean oocytes from maturing (Stricker et al., 2013). Unlike in mice, where the onset of oocyte maturation (germinal vesicle breakdown, GVBD) is blocked by cAMP and triggered by AMPK, oocytes of the marine nemertean worm *Cerebratulus* undergo GVBD in response to cAMP elevations and AMPK deactivation (Stricker, 2011). In addition these effects are observed only in the absence of the surrounding somatic cells (Stricker et al., 2010). These results also provide evidence for a novel GVBD-regulating mechanism involving AMPK deactivation by cAMP-mediated S485/491 phosphorylation and further highlight the highly species specific effects of AMPK in regard to oocyte maturation.

Male Germ Cells

α AMPK is present in male germ cells of oyster (Guévelou et al., 2013), chicken (Nguyen et al., 2014) and mammals (Hurtado de Llera et al., 2012a; Tartarin et al., 2012a; Cordova et al., 2014). In oyster, it is more highly expressed in male gonad than in female and its expression is more important during the first stage of gametogenesis when germ cells proliferate (Guévelou et al., 2013).

To traverse the female reproductive tract, it is essential that mammalian spermatozoa acquire the functional competence to achieve this objective in order to successfully fertilize the oocyte. These functional markers include motility, capacitation, hyperactivation and the acrosome reaction (Hurtado de Llera et al., 2015). It was recently demonstrated that AMPK protein is highly expressed in ejaculated boar and chicken spermatozoa (Hurtado de Llera et al., 2012a; Nguyen et al., 2014), and in mouse epididymal sperm (Tartarin et al., 2012a; Bertoldo et al., 2014a) and that it localizes in the head of the spermatozoon and in the midpiece of the flagellum (Hurtado de Llera et al., 2013). In the boar, pharmacological inhibition of AMPK lead to a reduction in motility (Hurtado de Llera et al., 2012a) while concomitantly causing changes in mitochondrial membrane potential, sperm plasma membrane fluidity and organization and acrosome integrity (Hurtado de Llera et al., 2013; Martin-Hidago et al., 2013). Similar results have been described in the chicken. These studies highlight the conservation of the AMPK function in sperm activity.

Interestingly, Hurtado de Llera *et al.* noted that the majority of studies to date that had studied sperm physiology as a function of AMPK had only done so under conditions where AMPK was inhibited (Hurtado de Llera et al., 2012b, 2015) or was genetically silenced (Tartarin et al., 2012a). Consequently they conducted a study to assess sperm physiology while activating AMPK indirectly. They observed under extended periods (24 h) of AMPK activation, boar spermatozoa had reduced motility, acrosome membrane integrity and organization and fluidity of the plasma membrane which was associated with an increase in lipid disorganization (Hurtado de Llera et al., 2015). As these processes are critical under the different environmental conditions experienced by spermatozoa when transiting through

the female reproductive tract to accomplish fertilization, it becomes obvious from studies carried out so far that an optimal level of AMPK activation is essential for regulating spermatozoa function (Hurtado de Llera et al., 2015).

Nakada *et al.* demonstrated that spermatogenesis is intimately linked to mitochondrial respiration (Nakada et al., 2006), and recently Pellicione et al. associated asthenozoospermia with abnormal mitochondrial ultrastructure (Pellicione et al., 2011). Therefore, it is likely that the motility disturbances observed in our α 1AMPK KO model and in the LKB1 KO model that presented with abnormalities in spermatozoa functionality and morphology (motility and head morphology) (Towler et al., 2008; Tartarin et al., 2012a), was directly linked to mitochondrial dysgenesis. Similarly, incubation of sperm from boar with an AMPK inhibitor, compound C, lead to a reduction in motility (Martin-Hidago et al., 2013). Surprisingly, a mouse model inactivated for an oxidative stress sensor protein like glutathione peroxidase 4 (Liang et al., 2009; Schneider et al., 2009) is described with structural abnormalities in spermatozoa analogous to those observed in α 1AMPK KO, suggesting that mitochondria dysfunction could affect oxidative stress oxidative status.

Diabetic and infertile men present with a decrease in anti-oxidant concentrations and an increase in ROS generation in their semen, even before cryopreservation, demonstrating that the sperm from this group are at increased risk of oxidative damage (Lewis et al., 1995; Garcez et al., 2010). Several studies have demonstrated that metformin can reduce the levels of oxidative DNA damage and afford anti-oxidant protection (Attia et al., 2009; Onken and Driscoll, 2010; Martin-Montalvo et al., 2013). Metformin has been shown to guard against diabetes-induced genomic instability in sperm cells and the bone marrow of diabetic rats (Attia et al., 2009), so we can hypothesize that the use of metformin in diabetic patients would have no negative effect in the integrity of spermatozoa. However, we cannot exclude consequences on the paternal genome for oocyte fertilization. For example, treatment of murine sperm with high concentration of metformin increased histone deacetylase activity (Bertoldo et al., 2014a). Curiously, treatment of sperm with known natural activators of AMPK such as resveratrol or a synthetic activator like metformin, present positive effects such as reduction in DNA damage and lipid peroxidation (Bertoldo et al., 2014a). A study in the wood frog (*Rana sylvatica*), a species with a high freeze tolerance, has revealed that AMPK was more activated in liver and muscle tissue, thus presenting AMPK as a molecule with cryoprotective properties (Rider et al., 2006). Subsequently modification of AMPK has been exploited in the freezing protocols of mammalian semen (Bertoldo et al., 2014a; Cordova et al., 2014). Metformin was used in mouse semen extender (Bertoldo et al., 2014a) and following thawing, spermatozoa showed an improvement in fertilization capability *in vitro*. This was associated with a reduction in the number of abnormal zygotes following IVF when mouse spermatozoa was frozen in the presence of metformin compared to controls (Bertoldo et al., 2014a). AMPK was also modulated in stallion semen extender where there was an improvement in sperm quality post-thaw (Cordova et al., 2014). As the reports of AMPK

presence in spermatozoa are growing in number, we believe it is reasonable to assume that AMPK activity is likely required for optimal mammalian spermatozoa physiology.

Germinal and Somatic Cells Interactions

Cumulus-oocyte Complex

Gap junction communication between cumulus cells and oocytes is crucial for oocyte meiotic maturation and to acquire full developmental competence (Gilchrist et al., 2004; Gilchrist, 2011; Li and Albertini, 2013). Such that maintenance of gap junction communication and delayed meiotic resumption have been shown to increase oocyte developmental competence (Thomas et al., 2004; Gharibi et al., 2013). Gap junction communication between the oocyte and the surrounding cumulus cells is established by the formation of bidirectional channels. The connexins family, which compose gap junctions is involved in oocyte/cumulus cell communication and allows passage of ions and small organic molecules. Loss of connexin 37 and connexin 43 in mouse oocytes or cumulus cells impaired fertility through inhibiting oocyte growth and folliculogenesis (Winterhager and Kidder, 2015). Electron microscopic analysis has shown that junctions between granulosa cells and oocytes are altered or absent as in connexin 37^{-/-} mice (Simon et al., 1997). In the mouse, deletion of $\alpha 1$ AMPK specifically in the oocyte lead to a reduction in connexin 37 between the oocyte and cumulus cells at the Metaphase II stage which was associated with reduced fertility following IVF, and suggests a reduction in gap junction communication (Bertoldo et al., 2015). Reductions in connexin 26 and connexin 37 expression were also described in a diabetic mouse model, where oocyte quality is poor (Ratchford et al., 2008). In an non-mammalian example, Alesutan et al., demonstrated that active AMPK decreased connexin 26 abundance in the cell membrane in xenopus oocytes (Alesutan et al., 2011), suggesting disparate regulation of gap junction communication by AMPK between species.

Deletion of $\alpha 1$ AMPK in oocytes leads to reductions in other proteins associated with intercellular communication within the cumulus oocyte complexes (Bertoldo et al., 2015). These include N-cadherin and β -catenin (markers for adherens junctions) and occludin (a marker for tight junctions) (Bertoldo et al., 2015). The cumulus-oocyte complex interacts with granulosa cells through adhesion junctions composed of proteins such as E-cadherin and N-cadherin (Rufas et al., 2000; Machell and Farookhi, 2003). Expression of N-cadherin for example, increases throughout maturation, fertilization and early embryogenesis (Ziv et al., 2002), and N-cadherin mediated cell contact is associated with the maintenance of meiotic arrest (Peluso, 2006). Deregulation of these proteins impacts oocyte maturation, fertilization and early embryogenesis (Ziv et al., 2002; Peluso, 2006).

Furthermore in the bovine, it was recently demonstrated that the transzonal processes (TZP) that traverse the zona pellucida transfer RNA molecules from cumulus cells to the oocyte (Macaulay et al., 2014). It was proposed that these TZPs are held in place by adherens like junctions (Macaulay et al., 2014) and are critical for oocyte developmental competence. During repair of lung capillary endothelium $\alpha 1$ AMPK promotes the development

of intercellular adherens junctions by binding with N-cadherin and contributes to repair (Creighton et al., 2011). This supports the notion that AMPK may have a critical role in oocyte developmental competence by maintaining open oocyte-somatic cell communication channels through at least gap, adherens and tight junctions. Taken together the literature supports the concept that AMPK plays a crucial role in maintaining metabolic and molecular intercellular coupling between the oocyte and its somatic cells and breakdown of this coupling results in reduced oocyte developmental competence.

Blood Testis Barrier

As in the cumulus oocyte complex, male germ cells are closely linked their support cells; the Sertoli cells during their maturation. Sertoli cells have an important role in the shaping of the spermatid head for example (Kierszenbaum and Tres, 2004). Different transgenic mouse models show that AMPK plays a role in intra-testicular communication. Absence of the upstream AMPK kinase, LKB1 reduced mature spermatozoa production associated with abnormal acrosome morphology and a defect in Sertoli cell polarity and testicular junctional complexes (Towler et al., 2008). Patients with Peutz-Jeghers syndrome present a similar phenotype with alteration of sperm production associated with modifications of tight junctions in the blood-testis-barrier (Ulbricht et al., 2007; Chen et al., 2012; Tanwar et al., 2012). The disruption of the $\alpha 1$ AMPK gene in the whole murine testis induced altered sperm morphology without presenting abnormalities in Sertoli cell nucleus polarization (Tartarin et al., 2012a). Nonetheless, transmission electron microscopy analyses have shown the presence of some disrupted Sertoli cell/elongated spermatid germ cell junctions (Tartarin et al., 2012a). Interestingly, a similar phenotype in sperm head or midpiece morphology has been already described in mice deleted for adhesion molecules like nectin-2 (Mueller et al., 2003) or Tslc1 (Surace et al., 2006). The fact that absence of the $\alpha 1$ AMPK gene lead to a mild phenotype in contrast to LKB1 could be explained by the activation of LKB1 through several other AMPK-related kinases present in the testis such as microtubule-associated protein/microtubule affinity-regulating kinases (MARK2) (Bessone et al., 1999) or SNRK (Jaleel et al., 2005) as a compensatory mechanism. This hypothesis is supported by the decrease in phosphorylation of MARK in testis in LKB1-KO mice (Kojima et al., 2007; Tanwar et al., 2012). The reduction and/or incorrect relocalization of markers of adherens junctions (β -catenin and N-Cadherin) (Kopera et al., 2010) and tight junctions (occludin and ZO-1) (Kopera et al., 2010) in Sertoli cells from LKB1-KO mice suggests a loss of contact with germ cells leading probably to alteration in germ cell shape (Kleymenova et al., 2005). Notably, altered β -catenin expression has previously been described to compromise Sertoli cell function and the maturation of germ cells and lead to infertility (Lee et al., 2005; Tanwar et al., 2010; Kerr et al., 2013).

The use of the AMPK activator (AICAR) can also influence junction complex integrity in rat Sertoli cells as has been described by Galardo et al. (2010). Rat Sertoli cells incubated with AICAR stabilized ZO-1 protein as observed by immunofluorescence (Galardo et al., 2010). The use of EGTA to

limit the free calcium concentration in culture medium induced a redistribution of ZO-1 between Sertoli cells. Addition of AICAR or adenosine, in the presence of EGTA permitted the rescue of ZO-1 distribution to normal conditions at the cell membranes (Galardo et al., 2010). As in oocytes, the cAMP/PKA pathway is modified in the absence of α 1AMPK in Sertoli cells, raising the question about the interaction between AMPK/cAMP signaling and the functionality of the blood-testis barrier permeability. Indeed, the cAMP signal has been described to be involved for the formation and maturation of male germ cells (Scobey et al., 2001), and can perturb junctions in rat Sertoli cells (Lui and Lee, 2005).

Interface between AMPK and Modification of Metabolism

Diet restriction is well known to promote longevity and reduce fertility in several species like *C. elegans*, *drosophila melanogaster*, birds and mammals. Diet restriction induces a negative energy balance which activates some energy sensors such as AMPK and the sirtuins which promote respiration and energy production by mitochondria. In mice, a deficiency in LKB1 or AMPK in mature Sertoli cells negatively impacts mitochondrial function, and has been associated with loss of quiescence and an activation of cell proliferation (Bertoldo et al., 2013). The association between nutrient availability, mitochondrial function and fertility has been already observed in invertebrates. In *C. elegans* the germinal stem cells regulate longevity through the TOR pathway (Arrantes-Oliveira et al., 2002), and similarly in *drosophila* TOR signaling is involved in the regulation of female germinal stem cell proliferation as a function of the availability of nutrients (Drummond-Barbosa and Spradling, 2001; Lafever and Drummond-Barbosa, 2005; Lafever et al., 2010; Shyh-Chang et al., 2013). These results are also observed in Sertoli cells where stimulation with an AMPK activator such as metformin or AICAR has consequences on lactate production and the increase in glucose transport (Galardo et al., 2007). One hypothesis of the action of metformin, is an indirect effect: an inhibition of the respiratory chain in mitochondria leading to an increase in lactate production, and in the AMP: ATP ratio inducing the activation of AMPK. On the other hand, the inactivation of α 1AMPK in Sertoli cells, reduces the expression of mitochondrial markers (cytochrome c and PGC1a) and the ATP content, and increases the lactate production (Bertoldo et al., 2013). The increase in lactate, in this case, could be due to a switch in the cell between energy production by respiration to the aerobic glycolysis. Thus, α 1AMPK deficiency enhances the Warburg Effect which can be associated with increased cell proliferation *in vitro* (Faubert et al., 2013). In addition, glycolysis could increase the allocation of glucose carbon into lipids and explain the increase in lipid vesicles. We cannot exclude that a modification in lipid metabolism in Sertoli cells has a consequence on germ cells. Indeed, some studies have described lipid transport from the Sertoli cells to the germ cells (Saether et al., 2003). Moreover, several recent studies using mice deficient in genes related to lipid metabolism, have described that the accumulation of excess lipid

droplets in Sertoli cells resulted in impaired spermatogenesis (Coussens et al., 2008). Therefore, a balance of lipid metabolism in Sertoli cells is essential for normal spermatogenesis (Selva et al., 2004).

Ratchford et al. have hypothesized that abnormalities in oocyte metabolism, such as that observed in diabetes, could potentially preprogramme the oocyte for unfavorable outcomes after fertilization (Ratchford et al., 2007). Furthermore, Wang et al. (2009) concluded that maternal diabetes results in numerous oocyte deficiencies. Glucose metabolism is essential for successful oocyte maturation and the recommencement of meiosis (Downs and Mastropolo, 1994). It is well known that mitochondria can influence the developmental competence of the oocyte (Thouas et al., 2004). Certainly mitochondria play a key role in cellular energy generation, the control of cell death (Perez et al., 2000) and the dynamic process of meiosis including DNA reorganization (Wang et al., 2009). In the case of diabetes, mitochondria are abnormally distributed around the spindle or in the oocyte cytoplasm (Wang et al., 2009). Ratchford et al. observed that under hyperglycaemic conditions, phosphorylated ACC, a downstream target of AMPK and phosphorylated AMPK were both decreased in diabetic oocytes, demonstrating decreased AMPK activity (Ratchford et al., 2007). Diabetic oocytes were also metabolically perturbed leading to altered AMPK activity. Interestingly, increasing AMPK with AICAR in these oocytes during the preovulatory phase corrected the metabolic and meiotic perturbations observed. For these crucial activities in oocyte maturation, mitochondrial redistribution, activity or dysfunction have been suggested as markers of oocyte quality and are strongly related to fertilization rates and embryo development (Van Blerkom, 2004; Wang et al., 2009).

During the last decade a variety of natural ligands and synthetic ligands have been shown to activate AMPK including resveratrol (Baur et al., 2006), sulforaphane (Choi et al., 2014), niacin (Thirunavukkarasu et al., 2006), berberine (Brusg et al., 2006), metformin (Zhou et al., 2001), and thiazolidinediones (Fryer et al., 2002). Some of these compounds have non-linear dose-response characteristics, such as that of hormesis and have the ability to inhibit the mitochondrial complex I at elevated concentrations that mimic diet restriction (Gems and Partridge, 2008). The hypothesis of hormesis lends weight to differences in phenotype associated with differences of metformin concentration. High metformin concentrations (approximately 5 mM) is enough to inhibit the respiratory chain complex 1 in mitochondria leading to an increase in the AMP/ATP ratio (El-Mir et al., 2000; Owen et al., 2000) and different metformin concentrations induce increases in oxidant defenses as well as an extension of lifespan (Onken and Driscoll, 2010; Martin-Montalvo et al., 2013). The difference in species sensitivity has been already observed as mouse tissue is 10 fold less sensitive than human tissue (Tartarin et al., 2012b).

Metformin is a good example for mimicking diet restriction, because in mouse liver, metformin has been shown to induce a similar transcription pattern to diet restriction especially (Dhahbi et al., 2005). However, similar effects have been described in *C. elegans* where metformin administration increases the

lifespan and produces several diet restriction-like phenotypes such as reduction in fecundity and a decrease in fat storage in animals which are fed *ad-libitum* (Onken and Driscoll, 2010). In *Drosophila*, metformin exposure for 7 days at 25 and 50 mM concentration increases significantly the number of eggs laid in contrast to untreated controls. But after 14 days of treatment, egg-laying in females on 25 mM metformin was similar to controls and at 50 mM of metformin the females laid significantly fewer eggs (Onken and Driscoll, 2010). Interestingly in flies, metformin targets AMPK and inhibits the TOR pathway (Kalender et al., 2010; Slack et al., 2012). From the reports to date, we can conclude that effects on fertility (increases or reductions in the number of egg laid depending the time and concentration of metformin treatment) remains partially understood and controversial (He and Wondisford, 2015).

SIRT1 is widely regarded as a critical regulator of energy homeostasis and is implicated in a wide variety of cellular processes including metabolic diseases, cancer, aging, and reproduction (Bordone and Guarente, 2005; Brooks and Gu, 2009; Haigis and Sinclair, 2010). Furthermore it is known to interact with AMPK (Fulco et al., 2008; Narala et al., 2008; Canto and Auwerx, 2009). We have recently provided evidence in the oocyte that α 1AMPK could be involved in chromatin remodeling, because we observed an increase in acetylation of H3 histone in oocytes from α 1AMPK knockout oocytes (Bertoldo et al., 2015). This was correlated, as expected with a reduction in histone deacetylase SIRT1 expression *in vivo*. *In vitro* Sirt1 has the ability to deacetylate histone substrates in a NAD⁺-dependent manner (Vaquero et al., 2004b) and hyperacetylation occurs when SIRT1 is knocked down (Vaquero et al., 2004b). Male mice deficient in SIRT1 present with altered germ cell maturation and increased DNA damage in germ cells (Coussens et al., 2008). Together, these data suggest that AMPK can modify oocyte proteins and histone acetylation status. These observations could

be linked to other reports such as those relating to the aorta and heart tissue where a decrease in AMPK and SIRT1 expression is associated with increased H3 acetylation (Bendale et al., 2013). Interestingly, acetylation of histones H3 and H4 appear to be linked to an overexpression of connexin 43 in a prostate cell line (Ogawa et al., 2005; Hernandez et al., 2006), and PGC1 α and p53 can modify their accessibility (Vaquero et al., 2004a; Wakeling et al., 2009; Nelson et al., 2012), possibly suggesting some level of control of intercellular communication and apoptosis. Interestingly, inadequate histone deacetylation causes changes in gene expression, which can lead to embryopathy in mice (Akiyama et al., 2006).

Conclusion

The involvement of AMPK in fertility control is conserved throughout several animal species from the oyster, *C. elegans*, *Drosophila*, birds and mammals. Its expression is present in different compartments of the ovary and testis and through the different stages of maturation of germ cells, germline stem cells to oocytes and spermatozoa. Apart from its classical functions on metabolism, proliferation and anti-inflammatory effects observed in the gonad, AMPK is also able to modulate steroidogenesis, and to impact morphology and normal nuclear maturation of germ cells through interaction of germ cells with their nurse somatic cells. Some mechanisms elucidated are directly linked with mitochondrial function and junctional proteins. Despite the possibility of different sub-unit combinations of AMPK, absence of only α 1AMPK leads to moderate failure of fertility in both sexes.

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Hypothalamic–pituitary–gonadal axis involvement in learning and memory and Alzheimer’s disease: more than “just” estrogen

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Accumulating studies affirm the effects of age-related endocrine dysfunction on cognitive decline and increasing risk of neurodegenerative diseases. It is well known that estrogens can be protective for cognitive function, and more recently androgens and luteinizing hormone have also been shown to modulate learning and memory. Understanding the mechanisms underlying hypothalamic–pituitary–gonadal axis-associated cognitive dysfunction is crucial for therapeutic advancement. Here, we emphasize that reproductive hormones are influential in maintaining neuronal health and enhancing signaling cascades that lead to cognitive impairment. We summarize and critically evaluate age-related changes in the endocrine system, their implications in the development of Alzheimer’s disease, and the therapeutic potential of endocrine modulation in the prevention of age-related cognitive decline.

Keywords: luteinizing hormone, menopause, ovariectomy, estrogen, testosterone, memory, Alzheimer’s disease

INTRODUCTION

As the baby boomer generation continues to age and advances in medicine have resulted in longer life expectancy, it is vital to consider the eminent risk of age-associated neurodegenerative diseases, such as Alzheimer’s disease (AD) and their effects on our society. The number of people diagnosed with AD is estimated to be over 13 million by 2050 (1). Furthermore, expense estimates for AD and dementia patient care in the United States alone are over \$180 billion per year, which makes dementia one of the most costly diseases (2). Not only will more individuals continue to be diagnosed and threatened with neurodegenerative disease, but their caregivers will also suffer more physical, mental, and economic burdens.

Senile plaques containing amyloid- β , neurofibrillary tangles comprised of aggregated tau and cell death in the hippocampal formation are the hallmark pathologies of AD (3). Clinically, AD is characterized by prominent deficits in memory and attention leading to deterioration of judgment, language skills, and spatial orientation as the disease progresses (4). While AD pathogenesis has been linked to oxidative stress, inflammation, and neuronal dysfunction, concrete evidence explaining disease pathogenesis is absent, compelling us and others to search beyond the hallmark pathologies for alternative molecular cues underlying AD pathogenesis (5).

Hormones are known to impact central nervous system (CNS) function and stability (6), and gonadal hormones have been extensively studied for their effect on cognition in elderly men and women (7). We believe that age-associated endocrine system dysfunction is a major factor in the onset and progression of neurodegenerative diseases. This is supported by research showing changes in gender and age-dependent reproductive

hormones increases the risk of AD, therefore implicating the hypothalamic–pituitary–gonadal (HPG) axis (8–14).

HYPOTHALAMIC–PITUITARY–GONADAL AXIS

The HPG axis is controlled by a negative feedback loop. In the healthy brain, the hypothalamus releases gonadotropin-releasing hormone (GnRH) into the median eminence, and then GnRH is transported via the hypophyseal portal system to the anterior pituitary where it acts on its receptor (GnRHR). Signaling from GnRHR leads to the production and secretion of the gonadotropins, including luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Once the gonadotropins are secreted into the blood stream, LH acts on its receptor in the gonads, which in turn stimulates the release of the sex steroids, androgens and estrogens. These sex steroids complete a negative feedback loop by inhibiting the release of GnRH.

Peripheral hormone levels have long been implicated in changes of behavior. Studies of the modulatory effects of hormones on cognition have led to the discovery that hormone receptors are expressed in the CNS (15, 16). Importantly, many of these hormone receptors are present in areas of the brain associated with learning and memory, such as the hippocampus. Crucial to HPG axis dysfunction and its connection to AD is that the rate of hormone synthesis varies throughout one’s life. With age, androgen production decreases gradually in men, while there is an abrupt diminution in gonadal secretion of estrogens in women. Besides, the dysregulation of the HPG axis caused by menopause may be due to a diminished ability of estrogens to inhibit the hypothalamus (17, 18). Interestingly, in addition to the negative feedback estrogens have on the HPG axis, estrogens can also produce positive feedback, which is necessary for fertility (19) and

is attenuated with age (20). This body of work strongly suggests that cognitive function may rely on the levels of sex steroids and gonadotropins, implicating HPG axis dysfunction as a factor in the strong correlation between aging and dementia.

ESTROGENS

Gender has been associated as an independent risk factor for AD. Females have a higher risk of developing AD as shown by age-adjusted odds ratios regardless whether familial or sporadic AD was acquired (21, 22). The higher prevalence and incidence of AD in women has been linked to the changes in reproductive hormones that occur during menopause. Therefore, women have become a main focus of many aging studies including the Women's Health Initiative Memory Study (9, 10). Endocrinological evaluations show lower levels of estrogens in women with AD (12, 23), thus encouraging hormone replacement therapy (HRT) to improve cognition and decrease the risk of AD in post-menopausal women (24–27). This theory is based on an abundance of evidence displaying the neuroprotective effects of estrogens observed in healthy cells. Estrogens signal through a multitude of mechanisms to induce axonal sprouting, regeneration, synaptic transmission, and the prevention of cell death (28). Estrogen receptors (ER) can activate production of brain-derived neurotrophic factor (BDNF), which has been shown to protect against ischemic injury *in vitro* and retain cognitive function as measured with passive avoidance in mice *in vivo* (29). Additionally, other *in vitro* studies have shown protective effects of estrogens from excitotoxicity by increasing the apoptosis regulator Bcl-2 (30). Moreover, estrogens have been shown to protect against oxidative stress induced by amyloid- β fibrils alone or in a complex with acetylcholinesterase, making estrogens a target for AD therapeutics (31). This hypothesis is influential in that the only current AD treatment is cholinesterase inhibitors, with which meta-analyses show a modest benefit (32).

Estrogen signaling has long been known to occur through nuclear receptor activity, but can also arise through membrane-associated ERs. The classic signaling pathway relies on nuclear ER α or ER β binding to estrogen response elements (ERE) in order to instigate transcription, therefore controlling gene expression. These long-term effects on gene expression are implicated in development as well as changes later in life. However, non-ERE-dependent signaling has been shown to rapidly produce effects of estrogens on neural processes. Membrane-associated ERs can activate PI3K/PLC, MAPK/ERK, and cAMP/PKA signaling pathways, which have been associated with neuroprotection (16). Importantly for the HPG axis, ER α mediates the negative feedback estrogens have on the hypothalamus without ERE activation to inhibit LH secretion through p21-activated kinase (33).

The neuroprotection evoked by estrogens was the basis of clinical trials looking for benefits on cognition. A randomized double-blind study found that 17 β -estradiol (E2) treatment and a consequent increase in serum estrogens provided no significant improvement in cognition (34). Furthermore, the Women's Health Initiative funded a study of 16,000 women that indicated HRT, with E2 and progestin, in fact increased risk of dementia. However, after controlling for treatment onset, it was found that initiation of HRT 10 or more years after menopause increases the risk of AD, while initiation of HRT during menopause lowers risk of AD

(9, 10). Supplemental research showed the treatment group in the Women's Health Initiative Memory Study had a higher incidence of breast cancer (35). This off target effect was pronounced with a combined E2 and progestin treatment, but is a major concern in HRT despite cognitive benefits observed. Overall, the clinical trials suggest that a critical period between menopause and HRT onset exists (36).

The results of E2 treatment have led to the proposal of the critical period hypothesis, which states that HRT is beneficial immediately after menopause, but benefits are lost the longer post-menopause is endured (36). The critical period hypothesis has also been demonstrated in ovariectomized rodents. The immediate post-operational treatment with E2 produces the well-characterized cognitive improvements, but a latency period of several months before E2 treatment ablates all benefits (37, 38). Along with the critical period hypothesis is the idea of the healthy cell bias. Estrogens may only be beneficial when administered to healthy neurons, and in order to potentially protect from neurological damage estrogens would need to be preemptively administered (39, 40). Conversely, once an unhealthy neurological state prevails estrogens signaling produces deleterious effects. Current hypotheses on the negative effects of estrogens in unhealthy neurological conditions include aberrant calcium signaling and mitochondrial dysfunction (41).

The reduced efficacy of estrogens downregulation of gonadotropins is evident in rodents after ovariectomy (42) and in post-menopausal women (43). Therefore, an alternate hypothesis that explains the lack of efficacy of HRT in older post-menopausal women is the inability of HRT to provide efficient negative feedback on gonadotropins. In this regard, LH is now known to influence cognition as well (44–48). Therefore, methodologically separating the roles of estrogens and LH on cognition is imperative to fully understand the impact of these hormones on cognitive decline and AD (37).

LUTEINIZING HORMONE

In conjunction with estrogens function in cognition, the loss of sex steroids leads to large increases in peripheral levels of LH. In aging women, a threefold increase in LH and a fourfold increase in FSH levels occur (49) while in aging men both hormones increase two to threefold (50). Until recently, the impact of changing levels of gonadotropins during reproductive aging was virtually ignored given the primarily peripheral role of gonadotropins on reproduction. However, mounting studies in humans, rodents, and *in vitro* demonstrate that LH and activation of its receptor may have an important role on cognitive function and neuronal plasticity.

In humans, increases in peripheral LH levels are correlated with decreased cognition in both healthy women (51) and men (52), and levels of LH and FSH have been shown to be significantly increased in AD patients compared to control (8, 13) in some but not all studies. For example, serum LH and FSH levels have been shown to remain consistent in female (12) and male (53) subjects regardless of dementia status, but by increasing the sample size, Hogervorst et al. (11) observed a trend toward high serum LH correlating with AD status in males. In a recent study, LH levels were correlated with amyloid- β levels, further implicating LH in development and progression of AD (14).

Human chorionic gonadotropin (hCG) and LH share a receptor (LHCGR) crucial for reproductive functions, such as testosterone production by Leydig cells and follicular maturation. A large body of literature describes the physiological and molecular role of LHCGR in reproduction (54). The isolation and purification of LHCGR revealed that it is a member of the G-protein-coupled receptor (GPCR) family (55, 56), and part of rhodopsin-like class A GPCRs expressed as several splice variants, all of which have leucine-rich repeats in the extracellular domain (54). Upon phosphorylation of LHCGR, G_{α_s} protein activates adenylyl cyclase and ultimately stimulates the cAMP/PKA and ERK pathways. Although LHCGR primarily signals through the G_s /cAMP/PKA pathway, it can independently mediate the activation of phospholipase C [PLC; (15, 57)].

Accumulating research shows LHCGR is atypically expressed in the CNS (58–60). LHCGR transcripts of 2.5 and 4.3 kb have been shown in fetal rat brain neurons and glial cells (61, 62). An 80 kDa band as seen by western immunoblot is evidence of protein expression in cultured rat neurons. Furthermore, time course studies show that day *in vitro* (DIV) 3 neurons have the highest levels of expression of LHCGR (61). A similar 80 kDa band is seen in immunoblots from glial cells which increase with an increase glial proliferation (62). Similarly, *in situ* hybridization has shown an abundance of LHCGR in the rat brain including the hippocampal formation, hypothalamus, cerebellum, choroid plexus, and ependymal cells of the ventricles and cortex (63). LHCGR expression has also been shown in the CNS of *Xenopus laevis*, where it plays a functional role in the reproductive behavior of courtship songs, therefore implicating a non-gonadal function of LHCGR in the CNS (64). Although many laboratories have researched LHCGR mechanisms in the gonadal organs, extra-gonadal receptor processes in rodents are just now beginning to be elucidated.

For example, early work shows that direct activation of the LHCGR with hCG drives cellular and functional changes. To this end, rat neurons cultured in the presence of hCG appeared to have increased numbers of neurite-bearing cells (61). The same treatment paradigm for glial cells showed an increase in prostaglandin D2 and a decrease in prostaglandin E2 (62). Importantly, prostaglandin D2 is known to curb the proliferation of glial cells; therefore, LHCGR activation in the CNS may be an important modulator of glial cell populations (62). Cell viability was increased with hCG treatment as observed through increased total protein content and decreased DNA fragmentation (61).

At a functional level, open-field behavior on rats injected with hCG either intraperitoneally (IP) or intracerebroventricularly showed a decrease in locomotor activity, but no increase in anxiety (65). Taste neophobia was overcome by intraperitoneal injections and intracerebroventricular injections of hCG, and hCG treatment did not evoke differences on T-maze behavior for the goal box, but a decrease was observed in the stereotypic behavior (65). This study shows that at a high dose hCG affects overall activity, which will supersede any findings related to learning and memory or fear. It has been proposed that at these high doses, hCG may be responsible for changes in sleep that occur during pregnancy since hCG is

present in cerebrospinal fluid during the first trimester and correlates with serum levels during the first and third trimester (65–67). IP injection of hCG in rats showed increases in both high and low amplitude sleep and a decrease in active awake phase via electroencephalography [EEG; (68)]. Similarly, peripheral hCG administration has been shown to decrease walking and increase resting in rats (68). It is important to note that during sleep LH pulses decrease in frequency but increase in amplitude (68).

Furthermore, studies using therapies aimed at downregulating peripheral LH show significant improvements in cognition and AD pathogenesis (44, 45). For example, in AD mice, downregulation of serum LH improves function in Y-maze and Morris water maze cognitive tasks and reduces amyloid- β immunoreactivity in the Tg2576 AD mouse (44). Animal studies utilizing GnRHR antagonists, antide and Cetrorelix, which also lowers serum levels of LH, also show cognitive improvements in spatial memory tasks in rats (46) and in non-transgenic models of AD (47, 69).

At a signaling level, studies show that downregulation of LH leads to activation of memory-associated cascades. CaMKII auto-phosphorylation, which is associated with hippocampal long-term potentiation (LTP), is downregulated after ovariectomy, but rescued with leuprolide acetate treatment (70). Furthermore, downstream targets of CaMKII, such as the phosphorylation of GluR1 subunit of AMPA are also activated by leuprolide acetate treatment (70). These changes in LTP-related cascades are one potential mechanism underlying the behavioral improvements observed with leuprolide acetate treatments *in vivo* (44, 48, 70). Interestingly, leuprolide acetate can also affect the synthesis of E2 from testosterone by modulating the transcription of p450 aromatase (70) suggesting functional benefits may be secondary to up-regulation of endogenous estrogens and downstream modulation of CREB and GSK3 β (48, 71, 72). Taken together, how LHCGR activation influences these cascades and leads to functional changes is a critical area of future study.

Interestingly, LH immunoreactivity has been demonstrated by radioimmunoassay as well as immunocytochemistry in the hypothalamus, amygdala, septal area, preoptic area, thalamus caudate nucleus, and hippocampus (73). Extracts from rat brain have shown that brain-derived LH has a similar chromatographic profile of pituitary LH and is active in the testis LH radio-ligand receptor assay as well as the interstitial cell testosterone secretion bioassay (74). Additionally, an adsorption significantly reduced rat brain LH immunoreactivity (74). Therefore, LH is both present and biologically active in the CNS. In support of these earlier studies, we have shown LH immunoreactivity in cognition-related brain regions in the 3xTg AD mouse model. Importantly, levels of brain-derived LH are decreased by ovariectomy, a state of high peripheral LH, and positively correlated with improvements in the Morris water maze (48). This suggests an inverse correlation between serum (peripheral) LH levels and CNS LH levels. Importantly, this inverse relationship between peripheral and brain-derived LH may explain the beneficial effects of hCG seen by Al-Hader et al. (61, 62) *in vitro* as well as the functional benefits and activation of LHCGR-dependent cascades by leuprolide acetate (48, 70).

ANDROGENS

Hypogonadism, a common feature of aging, may significantly contribute to both physical and cognitive decline in men, including the development of AD (75, 76). It has been shown that men with AD have lower levels of testosterone compared to non-AD patients (53). However, results have been mixed concerning testosterone replacement in healthy men. Testosterone replacement in non-demented patients with healthy gonadal function increased spatial cognition, verbal memory, and working memory (77–79). In contrast, testosterone replacement studies focusing on upper extremity strength or self-perceived health did not observe changes in cognition after 1 year of treatment (80, 81). Nevertheless, patients with mild cognitive impairment or AD have benefited from testosterone replacement. Spatial memory, constructional abilities, and verbal memory have all been shown to improve in cognitively impaired patients with short- (82) and long-term (83, 84) testosterone treatment. Meanwhile, one study suggests no difference between placebo and testosterone treatment (85), but the behavioral measures used may not have been sensitive to testosterone treatment (86), and, importantly, the testosterone injections may have been too interspersed to improve cognition. Trends in the literature suggest positive correlations between testosterone treatment and cognitive improvement warranting further investigation as a viable option for treating AD.

Essential to direct effects of testosterone on cognition, the androgen receptor (AR) is expressed in neurons of the hippocampus and amygdala (87, 88). Primary neuron cultures treated with testosterone show decreases in secretion of amyloid protein (89). *In vivo* studies show increased testosterone corresponds to decreases in β -secretase, an enzyme responsible for the cleavage of the amyloid- β precursor protein, and improves cognitive function in male double-transgenic mice (90). Effects of testosterone on CA1 spine synapses are independent from the AR since similar effects are seen in normal rats and AR mutants (91). In rats, rapid acting cytoplasmic androgen signaling in axons of the dorsal CA1 hippocampal neurons innervating the amygdala may be the most likely route for learning, especially in relation to environmental cues (88).

The AR binds testosterone or dihydrotestosterone (DHT) and translocates from the cytoplasm to the nucleus. Ultimately, AR signaling modifies gene expression, but like ERs, the ARs also have effects on intracellular signaling such as DHT's induction of CREB activation (92, 93). Extranuclear ARs are localized exclusively in asymmetric synapses (94) while ERs are found in both asymmetric and symmetric synapses (95). Redundancy may exist given the location of the ERs and ARs, but a unique function may exist for ER in symmetric synapses, which are typically inhibitory. Interestingly, for the action of ARs and ERs, testosterone can be converted into E2, which requires the experimental delineation of androgen and aromatized androgen metabolite action (96).

The conversion of testosterone into E2 by p450 aromatase (97) affords testosterone the ability to activate both ARs and ERs within the brain. Testosterone conversion can be controlled by aromatase inhibitors, therefore allowing experimental separation of testosterone pathways and the pathways of its aromatized metabolites (96). In a study of 60 healthy men aged 50–90, men treated with testosterone alone, and which consequently increases E2

levels from aromatase activity, displayed improvements in verbal memory. In contrast, when an aromatase inhibitor was administered along with testosterone, the improvement was no longer observed, suggesting that aromatization of testosterone is necessary for improvements in verbal memory (77). The individual actions of the sex steroids have separate roles in cognition; however, it is necessary to take neurological health into consideration during treatment. Testosterone metabolites improve cognition in healthy individuals, but as is evident from studies of estrogens, the neurological health of the patient may be a confounding factor.

Overall, a decrease in testosterone levels is associated with a higher risk of AD (11, 13, 14), and improvements in cognition have been observed with testosterone treatment (82–84). Testosterone signaling can occur directly through AR action or through the aromatization products acting on ERs but the bioactivity of the sex hormones may be a confounding factor (96). Increased levels of sex hormone-binding globulin (SHBG), which binds to sex steroids and reduces their bioactivity, have been linked to an increased risk of dementia in both men and women (98).

SEX HORMONE-BINDING GLOBULIN

Confounding the age-dependent attenuation of sex steroid signaling in the HPG axis is the ability of SHBG to reduce the bioactivity of sex steroids through binding and inhibiting action on their respective receptors. AD patients have increased levels of SHBG and, therefore, lower serum levels of bioactive sex steroids. The ensuing inverse correlation between cognition and SHBG is observed in both male and female AD patients (11, 98–101). SHBG levels and its inverse correlation with cognitive decline are important to consider for determining the efficacy of E2 and testosterone treatment. Importantly, SHBG levels may confound previous studies by deactivating the sex steroid treatments. Although further research is necessary, therapeutic control of SHBG would allow endogenous estrogens and androgens to remain bioactive and potentially reduce the risk of cognitive decline.

CONCLUSION

A myriad of research on the role of hormones in cognition has been undertaken and has led to many outlets for therapeutic potential to combat the cognitive loss associated with aging and AD. Epidemiological studies of the aging population show not only a gender difference in risk for AD, but also that HRT can have cognitive benefits. Prospective studies in humans and animals show that E2 treatment provides benefits to cognition when treatment onset is within the critical period, temporally close to menopause or hormone dysfunction. On a mechanistic level, it is well known that the levels of estrogens positively correlate with dendritic spine density in rodents as well as axonal sprouting, lending evidence for E2 treatment benefits being mediated through increasing plasticity. However, the diminished and deleterious effects of E2 treatment implemented outside of the critical period and the implication of this hormone in the development of breast cancer begets the search for hormone-based therapeutic strategies that go beyond E2 treatment. In this regard, one potential area of research is testosterone replacement. Work presented in this review demonstrates the beneficial effects of this hormone on learning and memory. However, effects of testosterone on cognitive function are primarily related

to the aromatization of testosterone into E2. As such, while testosterone may provide advantages through its ability to signal through AR and E2 receptors after its aromatization, this also poses issues at the level of dosing and off target effects. Taken together, while the actions of sex steroids have adverse effects, the improvements observed highlight the feasibility of HRT as a pharmaceutical treatment for cognitive decline throughout aging. However, aspects such as timing of replacement and clinical follow-up to mitigate important side effects must be carefully watched. Also important is the fact that secondary players, such as SHBG and p450 aromatase, that regulate bioavailability or production of steroids are key in determining the magnitude of effect and specificity of steroid hormone treatment. Whether these secondary players have a direct impact on cognition and neuroplasticity is unclear, and their determination is particularly important when interpreting the results of clinical trials using sex steroid replacement.

Lastly, we provide evidence that LH, a relatively unknown player in cognitive function and neuronal plasticity, is gaining support as a therapeutic target for age-related cognitive decline. Support for a role of LH in cognitive processes is highlighted not only by clinical data demonstrating high levels of peripheral LH are associated with cognitive deficits and AD, but also in pre-clinical studies. To this end, several investigators have now shown that lowering peripheral LH rescues cognitive decline in various aging and AD rodent models, and these treatments are associated with signaling important to synaptic plasticity. Importantly, a recent clinical trial in AD female patients shows benefits of downregulating peripheral LH (102).

Here, we propose the hypothesis that brain levels of LH become downregulated by increases in peripheral LH observed during ovarian function loss and cause cognitive dysfunction; this aspect, we hypothesize to be mediated through loss of LHR signaling in the brain. While the mechanisms in charge of downregulating brain LH production are unknown, support for this hypothesis is emerging based on data showing LHR expression and functionality in the brain as well as receptor actions associated with cognition-related signaling and neuroplasticity. Importantly, in our recent study, we observed a positive correlation between LH levels in the brain and learning and memory in the Morris water maze task. Studies directed at elucidating the specific role of the LHR in the brain are likely to shed light on the role of LH in cognition and neuroplasticity.

While in the last 30 years primary focus has been placed on studying the role of gonadal steroids on neuronal plasticity and function, we show here that aspects thought to be secondary or irrelevant, such as LH signaling, are important to fully evaluate the effects or lack thereof of gonadal steroids, and may potentially have direct roles and drive processes previously assigned to loss of steroid function. As such, a comprehensive study of HPG axis hormones is necessary in clinical and pre-clinical work and likely to be more effective in providing novel therapeutic targets and strategies for cognitive dysfunction and AD.

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Structure–function relationships of glycoprotein hormones and their subunits' ancestors

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Glycoprotein hormones (GPHs) are the most complex molecules with hormonal activity. They exist only in vertebrates but the genes encoding their subunits' ancestors are found in most vertebrate and invertebrate species although their roles are still unknown. In the present report, we review the available structural and functional data concerning GPHs and their subunits' ancestors.

Keywords: glycoprotein hormones, luteinizing hormone, follicle-stimulating hormone, thyroid-stimulating hormone, evolution, molecular, structure–activity relationship

INTRODUCTION

Glycoprotein hormones (GPHs) are the most complex molecules with hormonal activity. They include three pituitary hormones, the gonadotropins follicle-stimulating hormone (FSH; follitropin) and luteinizing hormone (LH; lutropin) as well as thyroid-stimulating hormone (TSH; thyrotropin) (1). Only in primates (2) and equidae (3), a chorionic gonadotropin (CG) is also secreted by the placenta.

The GPHs exist only in vertebrates and appeared during evolution along with the pituitary. Nevertheless, genes coding for molecules related to GPHs subunits were identified in all vertebrates studied and in most invertebrates (4–6).

The gonadotropins FSH and LH play a central role in vertebrate reproductive function (7, 8) as they convey the integrated central information from the hypothalamic–pituitary complex toward gonads in both males and females. Indeed, internal (mainly endocrine) and external (photoperiod, congeners) information are integrated at the hypothalamus level by pulsatile gonadotropin-releasing hormone (GnRH) secretion. In mammals, GnRH is released by GnRH neurons in the portal hypothalamic–pituitary system through which it enters into the anterior pituitary. In fishes, GnRH neurons release GnRH directly into the pituitary. In all cases, GnRH stimulates the secretion of both gonadotropins FSH and LH by the anterior pituitary but their secretions are also differentially modulated by gonadal feed-backs through the action of steroid hormones and protein factors.

The TSH is also secreted by the antehypophysis but under the control of the hypothalamic neuropeptide thyrotropin releasing hormone (TRH) and is modulated by thyroid feed-back through the action of thyroxine (T4) or tri-iodo-thyronine (T3).

The placental gonadotropins (hCG in human; eCG in the mare) are secreted by trophoblast cells under no known control by any releasing hormone.

In the present paper, we will consider the structure–function relationships of GPHs and of their receptors (GPHRs) to better understand their interactions and the subsequent steps in their target cells stimulation.

STRUCTURE OF GLYCOPROTEIN HORMONES AND THEIR ANCESTORS

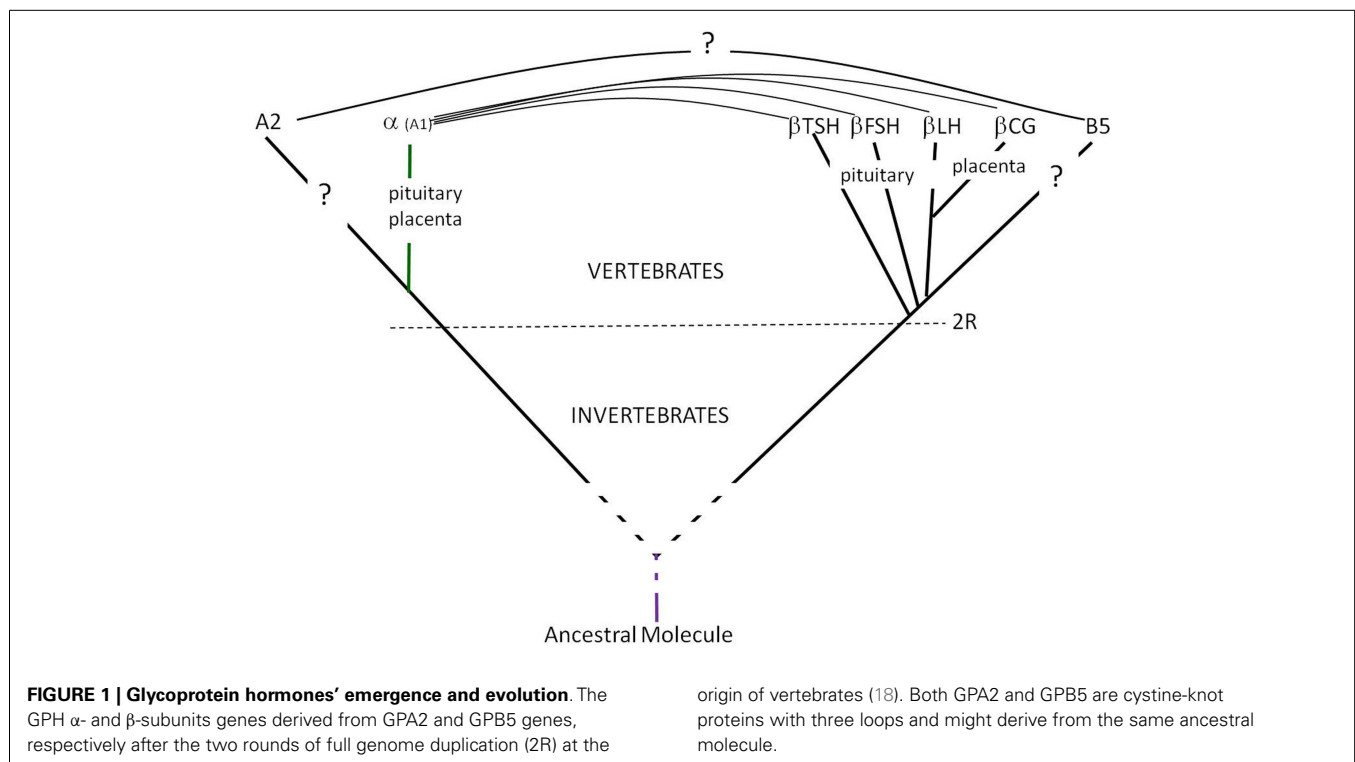
Since 1971, GPHs are known to consist of two different glycoprotein subunits, called α and β , that are non-covalently associated (9–12). This heterodimeric structure has been known for a long time to be mandatory for their respective biological functions.

The saccharide part in GPHs represents as much as 20–45% of their total mass (11, 13, 14) and has been shown to be indispensable for their *in vivo* bioactivity (15, 16). It is therefore important to get as much information as possible concerning both their polypeptide and polysaccharide portions as to decipher their respective roles.

More recently, genes encoding for proteins related to the GPH α and β subunits were found in both vertebrates and invertebrates and were named GPA2 and GPB5, respectively (4, 6, 17) and are considered as the molecular ancestors of GPH subunits (**Figure 1**). Recombinant GPA2 and GPB5 have been produced using plasmids encompassing the coding regions from these genes. These recombinant molecules were characterized using various immunoassays and *in vitro* bioassays. The natural GPA2 or GPB5 proteins have never been isolated but were detected in adult rat pituitaries by immunohistology and western blotting using antibodies raised against the recombinant proteins (6). The putative GPA2/GPB5 heterodimer has been described to exert thyrostimulating activity (i.e., the name thyrostimulin coined for it) (6).

POLYPEPTIDE PART

The GPHs α - and β -subunits are encoded by different genes (i.e., they do not originate from post-translational



proteolytic maturation of a unique precursor like insulin subunits).

The α -subunits of all GPHs in a given species are encoded by a same and unique gene that is expressed in pituitary gonadotrope and thyrotroph cells of all vertebrates as well as in chorionic syncytiotrophoblastic cells of primates and equidae. Therefore, the GPHs α -subunits all exhibit the same amino-acid sequence in a given species.

By contrast, the β -subunits are different and specific for each hormone. Therefore, there are at least three genes encoding β -subunits in all vertebrate species, namely FSH β , LH β , and TSH β . In the human species, there are not only one but several genes encoding the hCG β subunit.

The two subunits are thus co-translated and they non-covalently combine in the endoplasmic reticulum of gonadotrope (FSH, LH), thyrotroph (TSH), or trophoblast (CG) cells. No information is available for the time being concerning the natural GPA2 and GPB5 proteins.

Primary structure

All GPH subunits sequences as well as those of GPA2 and GPB5 exhibit a signal peptide at their N-terminus indicating that all these molecules are secreted glycoproteins.

The common α -subunits (GPA1) of mammalian GPHs after excision of their signal peptide exhibit 92 or 96 amino-acid sequences among which 10 are cysteine residues. Since no cysteine residue is in the reduced state, the α -subunits possess five disulfide bridges (Figure 2). In other species, the matured α subunits also count approximately 90–100 amino-acid residues (19).

The amino-acid sequences of α -subunits are very well conserved among vertebrate species and in particular the positions of the 10 cysteine residues. The residues are found in groups of

two or three along the sequence leaving three sequence portions without cysteines that are expected to form loops.

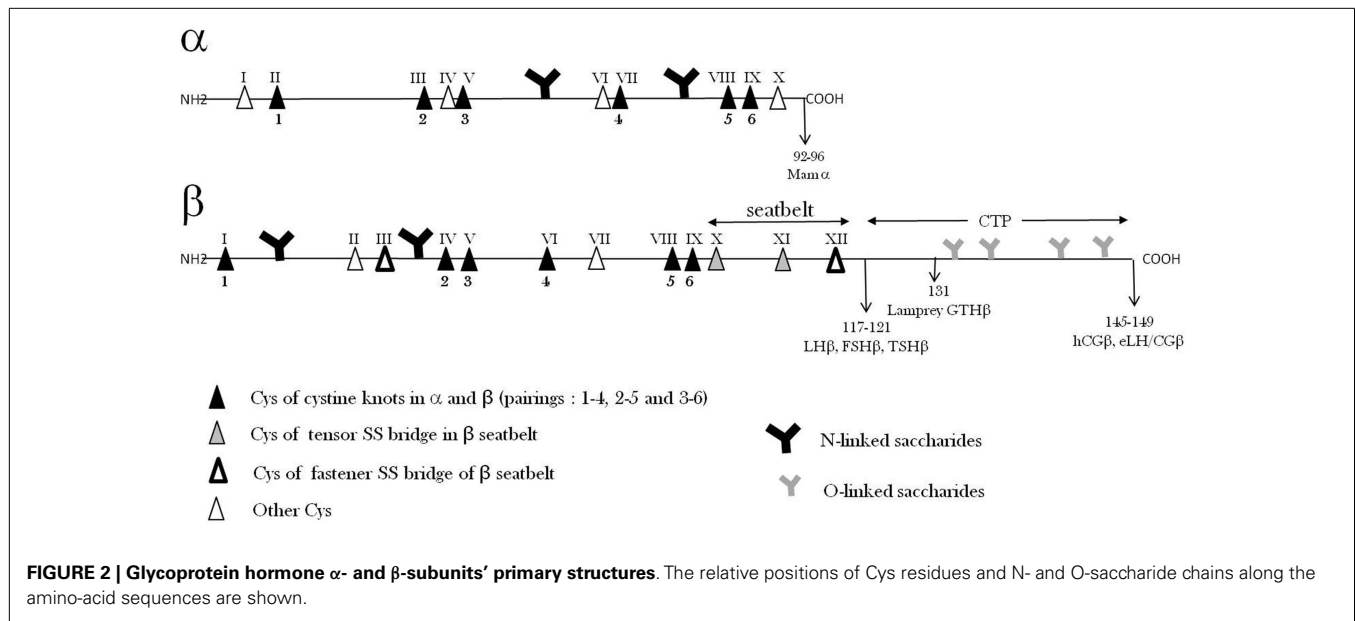
In the α -subunits, two potential N-glycosylation amino-acid sequences (Asn–X–Ser/Thr) are found and both of them are indeed occupied by oligosaccharide chains.

The amino-acid sequences of common α -subunits from all vertebrates exhibit a fairly high percentage of identity. This is well illustrated by the fact that it is possible to recombine these α -subunits with β -subunits from the other GPHs and from phylogenetically distant species (20, 21).

The GPA2 amino-acid sequences derived from the gene sequences in numerous species indicate that there are two potential N-glycosylation sites but one is not at the same location as in α -subunits.

The specific β -subunits (FSH β , LH β , TSH β , and CG β) polypeptide sequences exhibit around 105–150 amino-acids (19). Although different, the β -subunit amino-acid sequences exhibit large similarities which are probably largely due to their main common characteristic which is to associate with a common α -subunit. The conserved sequences in the β -subunits are important for (1) direct interaction with α and (2) similar global folding.

All β -subunits possess 12 cysteine residues which are all implicated in the six intra-chain disulfide bridges. In spite of their different sequences that determine the specificity of the different hormones, the β -subunits share a number of common features. In particular, the positions of the 12 cysteine residues in their sequences are highly conserved so that they are expected to share a common global folding (see below). The repartition of cysteines along the amino-acid sequences of β -subunits also leaves sequences without cysteines that are expected to form three loops (L1, L2, L3).



Some mammalian β -subunits (hCG β , eCG β , and eLH β) possess an extension of approximately 30 amino-acid residues at their C-terminus that is called carboxy-terminal peptide (CTP). This extension has of course occurred independently in primates and equidae through stop codon frameshift mutations leading to readthrough of the previously untranslated 3' downstream nucleotide sequence (22, 23).

The GPB5 amino-acid sequences derived from their gene sequences show that this molecule is shorter than β -subunits by approximately 15–20 residues and misses cysteine residues III and XII present in β -subunits (Figure 2).

All GPB5 β -subunits possess one or two potential N-glycosylation amino-acid sequences (Asn–X–Ser/Thr), which are found in conserved positions among the different species. The GPB5 amino-acid sequences derived from the gene sequences in numerous species indicate that there is one potential N-glycosylation site but at a different location than in β -subunits.

Secondary structure

The circular dichroism (CD) analyses of GPHs had shown a long time ago that they exhibit a limited amount of periodic (secondary) structures: only ~4–8% α -helix and ~30% β -sheet and β -turn (24–30). From these data, the rest of the molecule was expected to be non-periodic. Interestingly, the combination of subunits was paralleled by an increase in β -structure as determined by CD (31, 32).

The three-dimensional (3D) structure of hCG determined by X-ray diffraction (33, 34) confirmed the low amount of α -helix but the proportion of β -sheet and β -turn appeared more important than expected from CD data. Indeed, a very large β -sheet involves sequences from both subunits and this structure is suspected to be important for their heterodimerization. It is likely that the increase in β -structure as determined by CD is due to the formation of this common larger β -sheet involving peptide portion from both subunits.

A possible explanation for the difference in β -structure proportion as determined by CD and X-ray diffraction is that the first one is carried out in solution whereas the second is performed in crystals. It has been shown by hydrogen isotope exchange that gonadotropins exhibit a highly mobile conformation in solution (35). It is likely that this structural dynamics observed in solution is restrained inside the crystal.

Tertiary structure

The folding of hCG subunits (tertiary structure) has been determined by X-ray diffraction (33, 34, 36). The determination of this 3D structure has also permitted to find out the pairings of cysteines in each of the 11 disulfide bridges (5 in α ; 6 in β). An outstanding structural feature has been discovered concerning disulfide bridges: three of them in α and three of them in β form a cystine-knot with similar pairings (Figure 2) (37–39). In each cystine-knot, a disulfide bridge passes through the frame formed by two other parallel disulfide bridges joining two amino-acid sequence stretches. Such a structure, found only in a limited number of proteins including TGF β family (TGF β , BMPs, activin, inhibin, etc.) (39), is highly stable and forms the core of the 3D folding of the two subunits (37, 38). In the 3D structure, the three loops in α -subunits and the three loops in β -subunits can be observed that extend from the cores of each subunit.

Quaternary structure/heterodimerization

The quaternary structure of glycoprotein consists of heterodimerization of one α -subunit (common) with one of the specific β -subunits. In the association of the two subunits, the L1 and L3 loops of one subunit is aligned with the L2 loop of the other (33, 34). As previously stated, a large β -sheet structure formed by complementary regions from the α - and β -subunits is probably responsible for their non-covalent association.

Association–dissociation equilibrium constants of GPH subunits were found to be around 10^{-7} – 10^{-6} M (40, 41). These values

are much higher than the GPH physiological circulating concentrations (10^{-11} – 10^{-9} M) and therefore the hormones should be totally dissociated and inactive at these concentrations. In fact, it has been shown a long time ago that GPH integrity *in vivo* is a kinetically regulated process (41), i.e., the equilibrium is reached very slowly giving enough time for the heterodimer to exert its action. This view has been validated later when the “seatbelt” structure was discovered. Indeed, an outstanding feature of this quaternary structure is that a sequence portion of the β -subunit, from Cys β X to Cys β XII, wraps around the α -subunit, forming a “seatbelt,” which is fastened by a disulfide bridge between Cys residues β III and XII (**Figure 2**) (33, 34).

It is noteworthy that the seatbelt sequence as well as the two cysteines forming the fastener in β -subunits are missing in the GPB5 sequences rendering unlikely the existence of a stable GPA2/GPB5 heterodimer. In line with this view, recombinant GPA2/GPB5 heterodimers were evidenced by SDS-PAGE and Western blotting only after chemical cross-linking (6, 42, 43) indicating that the recombinant GPA2/GPB5 heterodimer is highly unstable.

It is tempting to postulate that GPA2/GPB5 heterodimerization is required for bioactivity as it is the case for GPH α and β subunits. The princeps paper (6) described thyrostimulating activity for the GPA2/GPB5 heterodimer and further studies led to the same (44) or other proposals for GPA2/GPB5 bioactivities in mice (45) and insects (46, 47). Nevertheless, it was not clear from these papers whether GPA2 and GPB5 were chemically cross-linked in GPA2/GPB5 heterodimers used in the bioactivity studies. Thus, it cannot be ruled out that GPA2 and/or GPB5 exert biological functions of their own (48, 49).

Cooperative folding

The thermodynamics of GPH subunits combination has been studied by microcalorimetry and the loss of cooperative folding was observed only at high T_m , i.e., above 70°C (50). Short-term incubations (5 min) of different GPHs at temperatures between 4 and 86°C followed by sandwich ELISA to detect residual heterodimeric molecules, led to similar data (51). The conservation or restoration, of the 3D structure at fairly high temperatures was also confirmed by measuring residual bioactivity in cell-culture assays.

These data indicate that the presence of one cystine-knot in each of the two subunits together with the fastened β -subunit “seatbelt” around the α -subunit, ensure very stable tertiary quaternary structures even if it has been shown to be highly mobile by hydrogen isotope exchange of peptide protons (35).

Kinetics and equilibrium of seatbelt fastening

The β -subunit “seatbelt” around the α -subunit fastened by the III–XII bridge does not influence the K_d of the subunits combination ($\sim 10^{-6}$ M) but considerably lowers the rate of subunits dissociation at physiological hormone concentration ($\sim 10^{-11}$ – 10^{-9} M). The two disulfide bridges in the seatbelt, β III–XII (latch) and β X–XI (tensor) appear to be involved in its opening and closing during the heterodimer $\alpha\beta$ formation in the endoplasmic reticulum (52–54). Indeed, in the presence of a mixture of oxidized and reduced glutathione mimicking the endoplasmic reticulum redox potential, the dissociation rate of subunits is considerably

accelerated. In contrast, at the much more oxidizing redox potential corresponding to that of serum, the seatbelt remains fastened and the dissociation rate of subunits is extremely slow (40).

Therefore, the hormone remains dimeric, and active, during its time of presence in the circulation even if its K_d is unfavorable (1000–100,000-fold higher than hormone circulatory concentrations).

It is important to point out that GPB5 protein sequences derived from their gene sequences lack the polypeptide portion forming the “seatbelt” as well as the two cysteine residues (III and XII) forming the “seatbelt fastener” (4, 55) in all GPH β sequences. This argues against the existence of stable secreted GPA2/GPB5 heterodimers (38, 48).

POLYSACCHARIDE PART

The mammalian GPHs contain from 15 to 45% saccharide in mass. Polysaccharide chains are only N-linked in most of these hormones (two in α and one or two in β ; **Figure 2**) (13). However, a few of them (hCG, eCG, and eLH) also possess O-linked saccharides on their CTP extension in their β -subunits (13, 56) (**Figure 2**). These sugar moieties confer high solubility, increase apparent molecular mass in SDS-PAGE, and charge polymorphism of the GPHs. These physico-chemical properties also convey numerous important functional properties to them. It is therefore important to consider them here.

N-linked saccharide chains

The positions of N-linked saccharide chains are determined primarily by the presence of potential N-glycosylation amino-acid sequences (Asn–X–Ser/Thr). Saccharide chains are transferred “en bloc” in the endoplasmic reticulum to these Asn residues in the polypeptide chains during the course of their translation. At this step, the N-linked saccharide chains are of the immature type, i.e., high-mannose and glycosylated. Interestingly, these high-mannose type N-glycans have been described to possess chaperone-like function during protein folding in endoplasmic reticulum (57) and accordingly they facilitate correct disulfide bond pairing (58). Correct folding is assessed by a quality control system consisting of chaperone proteins such as calnexin, BiP, and/or Grp94 (59). This quality control also involves the removal of the three glucose residues from these chains before the transfer to the Golgi apparatus.

Maturation of the N-linked chains then occurs in the Golgi apparatus. It consists in partial mannose removal by two mannosidases, addition of GlcNAc, Gal, and sialic acid residues by specific enzymes. The enzyme assortment in different cell types can differ so that N-saccharide chains can differ from one cell type to the other. This is particularly obvious for equine LH and equine CG that are encoded by the same α - and β -subunit genes.

These hormones are synthesized in the pituitary and placenta respectively and although they share the same polypeptide chain sequences, they exhibit largely differing sugar moieties (56). The N-saccharide chains are of complex or hybrid types and possess one to four antennas which are either completed up to a terminal sialic acid residue or not.

Concerning GPA2 and GPB5, only recombinant molecules and no natural ones have been available for structural studies.

Therefore, it is possible to spot potential N-glycosylation sites in their sequences but the presence or absence of saccharide chains at these sites can only be checked in proteins synthesized in heterologous systems (CHO, HEK, Sf9 cells). The analysis of GPA2 sequences shows the presence of two potential glycosylation sites at the same locations in vertebrates and the urochordate *Ciona intestinalis* and of only one at a completely different location in the cephalochordate *Branchiostoma* and in protostomes. This observation reinforces the view that among chordates, urochordates are phylogenetically closer to vertebrates than cephalochordates (60). Concerning GPB5, there is one potential N-glycosylation site in all the analyzed deuterostomes (mammal vertebrates, *Ciona* protochordate, *Strongylocentrotus purpuratus* echinoderm) but at different locations along their respective amino-acid sequences. By contrast, none was found in the protostome sequences.

O-linked saccharide chains

O-linked saccharides are found in GPH heterodimers possessing a CTP at the C-terminus of their β -subunits (hCG, eCG, and eLH). Four O-linked saccharides are found in the hCG CTP whereas as much as twelve of them can be found in eLH and eCG CTPs. The numerous O-saccharides found in the CTP are thought to impede compact folding of the polypeptide chain and to keep it extended (61).

The α -subunit is synthesized in excess relative to the β -subunits in the pituitary, and interestingly, it has been found that the excess free α molecule partly undergoes an O-glycosylation at its Thr43 residue (51, 62–65). For hCG, this O-glycosylation at Thr43 is a late event in the secretory pathway that occurs in the Golgi apparatus whereas $\alpha\beta$ combination occurs earlier in the endoplasmic reticulum and therefore O-glycosylation of free α plays no role in the heterodimer assembly.

Interestingly, the free α molecule has been found to play paracrine roles in the pituitary and the placenta (63, 66–70).

The molecular compositions of O-linked saccharides are variable (71). Some of them are composed of only three saccharide units whereas others are extremely long with poly-lactosamine extensions. These long extensions in addition to the unfolding effect of multiple O-saccharide sites on the polypeptide chain are responsible for the high apparent molecular weights of the CTPs in hCG and eCG.

Isoforms

The differences in their number of antennas and in their completion render N-saccharide chains extremely heterogeneous in terms of mass and charge. Probably hundreds of hFSH isoforms might exist due to all possible variants of their four N-saccharide chains (72). The number of potential isoforms is even higher in the case of GPHs with both N- and O-saccharide chains such as hCG, eCG, and eLH but less variability was observed in hCG N-linked carbohydrates than in those in hFSH.

Due to this mass and charge heterogeneity of their saccharide chains, GPHs exhibit an important polymorphism in electrophoresis or chromatofocusing. Such a large polymorphism makes the physico-chemical characterization of GPH preparations very difficult. This is true for natural as well as for recombinant

hormones which must be produced in eukaryotic cells in order to be glycosylated.

Polymorphism due to size and charge heterogeneity of their saccharide moieties has also been found to vary as a function of physiological situations (menstrual cycle, gender, age) (73, 74) and occasionally as a consequence of pathological situations.

In the case of TSH, it has been recently reported in the mouse that the hormone produced by the pituitary pars-distalis (PD-TSH; ~35 kD) essentially bears biantennary and sulfated N-linked carbohydrate chains whereas that produced by the pituitary pars-tuberalis (PT-TSH; ~40 kD) bears sialylated bi-, tri-, and tetra-antennary carbohydrate chains (75). It is reported by these authors that only PD-TSH stimulates thyroid hormones secretion whereas PT-TSH acts only on the hypothalamus to regulate seasonal physiology and behavior. The different glycosylations of these two tissue-specific TSH isoforms are thus responsible for their differing functional properties *in vivo*. This difference is not attributable to differing TSH receptors (TSHRs) in the thyroid and hypothalamus but to different affinities toward IgG and albumin leading to differing spatial distributions of the two isoforms (75).

STRUCTURE AND IMMUNE PROPERTIES

Immunologic properties of GPHs have been used for a long time in order to set up immunoassays to measure their concentrations in blood in normal physiological conditions as well as in pathological situations. Also, GPHs can promote the production of antibodies that can adversely affect their function. It is thus of interest to consider these properties for a better understanding of their physiological roles and in view of a better control of their activity in clinical situations where they are injected to patients.

For the setting-up of immunoassays, it is important to raise antibodies that can distinguish the different GPHs. Therefore, these antibodies are most often directed against the specific β -subunits. Nevertheless, it is often more efficient to raise antibodies against the heterodimer and to select either those that are specific of the conformation taken by the β -subunits when associated with the common α -subunit or those that recognize epitopes contributed by both subunits in the heterodimer. The free α -subunit is not very immunogenic but a number of antibodies against the heterodimers recognize epitopes in the associated α -subunit.

Monoclonal antibodies against each subunit are particularly valuable for the setting-up of sandwich ELISAs that specifically detect the heterodimers and differentiate them from free subunits.

HALF-LIFE AND ELIMINATION OF GONADOTROPINS FROM CIRCULATION

The contribution of carbohydrate chains to GPHs half-lives has been known for a long time (76–78). Their size and charge both contribute to their maintenance in the circulation. There are two main routes of elimination of gonadotropins from blood: liver capture (78) and more prominently kidney glomerular filtration (79–81).

For bulky plasma glycoproteins, half-life is essentially determined by their hepatic capture (76). Indeed, the -Gal-NANA end of N-saccharide branches can more or less rapidly be desialylated so that the Gal residue becomes exposed and recognized by the hepatic Gal receptor (82). This leads to removal of the

glycoproteins from circulation. Experiments in which hepatic circulation in piglets is partly bypassed from the hepatic portal vein to the vena cava do not show any significant increase in LH half-life (79). It is therefore likely that the liver is not the most important route of GPH elimination from circulation. The apparent molecular weights of GPHs are far below the glomerular filtration limit (~60 kD) and therefore they are readily eliminated through urine (except eCG). The high concentrations of bioactive hCG in pregnant women's urine and of bioactive LH and FSH in post-menopausal women's urine clearly demonstrate that these hormones are readily filtrated through renal glomerulus without major alteration of their structure. Accordingly, the half-lives are extremely short (5–30 min) for GPHs without CTP such as hFSH and hLH.

Also in keeping with this, a mutated recombinant hFSH with four additional N-linked glycan chains was found to exhibit a longer half-life and consequently a higher *in vivo* activity (83). Nevertheless, liver mannose receptors appear to be involved in the removal from circulation of glycoprotein with glycans terminated by β 1,4-linked GalNAc-4-SO₄ such as in LH mainly (84, 85).

Placental GPHs possessing a CTP with long O-carbohydrates exhibit longer half-lives (1.5–2.5 days) than pituitary GPHs without CTP (5–30 min). Indeed, the hydrophilic long saccharide chains borne by the CTP are very bulky and negatively charged because of their terminal sialic acid residues. These properties lead to lowered glomerular filtration because of the size and negative charges of glomerular pores. This explains why eCG which possesses the greatest and most acidic saccharide chains on its CTP is not found in urine in contrast to LH, FSH, and even hCG as mentioned above.

This unique property of β CTP has been exploited by fusing hCG β CTP to the hFSH β subunit sequence in recombinant hormones in order to increase its half-life in circulation (86–88) (**Figure 3**). In further works, β CTP was used as a tether between subunits to produce various single-chain GPHs (51, 89–93).

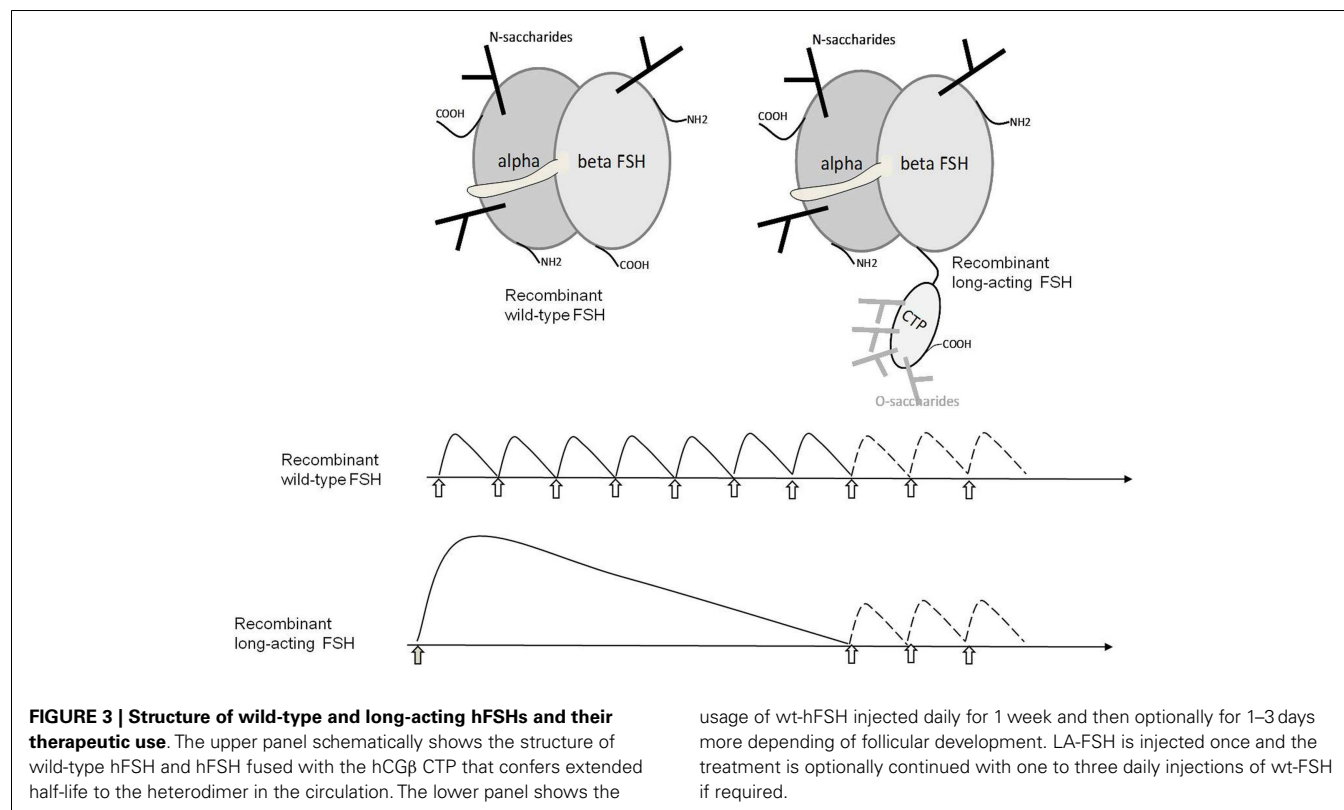
MOLECULAR MECHANISMS OF ACTION OF GLYCOPROTEIN HORMONES

The very first step in GPH action is their binding to specific receptors (GPHRs) at the plasma membrane level of gonadal cells. GPHRs are seven transmembrane domain (7-TMD) receptors with a very large extracellular domain (ECD) containing numerous leucine-rich repeats (LRR) (94) with a horseshoe shape that accommodate GPH binding.

Following high-affinity and specific binding with the receptor's ECD, an interaction with the 7-TMD must occur to promote a transconformation that is detected by the intracellular partners, essentially the heterotrimeric G-protein Gs but also, in certain situations, other pathways (95).

BINDING TO SPECIFIC RECEPTORS

In spite of their respective large similarities, GPHs specifically interact with their cognate GPHRs. This is true at least when considering hormones and receptors from the same species. Indeed, eLH and eCG are known to exhibit FSH activity in addition to their LH activity in all species except their own (horse) and chicken. Moreover, hCG exhibits low but significant TSHR binding activity in addition to its strong LHR binding activity even in its own species (human).



It has been known for a long time now that both the GPH common α - and the specific β -subunits participate in receptor binding (95, 96). It appears from X-ray diffraction studies of the FSH–FSH ECD complex that the β -subunit's seatbelt and the two neighboring regions in the α -subunit form the hormone surface that interacts with the receptor's ECD (36, 97, 98). The involvement of the common α -subunit in receptor binding suggests an important similarity in the interaction mechanisms. From comparative data, we were the first to propose a long time ago the “negative specificity” model. In this model, the α -subunit contribution to binding is only high-affinity whereas that of the β -subunit's seatbelt is to control specificity by inhibiting hormone binding to the “wrong” receptors (99, 100). It is interesting to point out that GPB5 that lacks this seatbelt region not only would lead to unstable GPA2/GPB5 heterodimers but also would lack this specificity mechanism leading to their potential binding to all GPHRs. Since TSHR is the receptor with the lowest specificity as it also binds hCG (101, 102), this may explain why GPA2/GPB5 binds only to it and not to FSHR and LHR.

The crystal structure of the FSHR ECD in complex with FSH (36, 95, 103) indicates that FSH establishes contacts with some β -strands of the concave inner surface of the leucine-rich repeat domain (LRRD) of the ECD (94). This paper also report dimerization of the FSH/FSHR ECD complex in agreement with previous data showing that recombinant GPHRs functionally defective either in their ECD or 7-TMD can complement and transduce the hormonal signal when co-expressed in the same cells (104, 105). More recent data suggest a trimerization of FSHR upon FSH binding (106) and an activation mechanism involving a FSH transconformation allowing its direct interaction with the FSHR sulfated Tyr residue at position 335 (95).

In the TSHR, the hinge region does not only serve for stabilizing the receptor ECD LRRD but it also participates in TSH binding (107).

The position of certain N-saccharide chains can affect specificity of binding. Indeed, it has been shown that eCG and eLH do exhibit FSH activity in all species tested so far but not in their species of origin (horse) and also very weakly in chicken (108). As mentioned above, the presence of an extra saccharide chain at the same position (Asn 268) in the FSH receptors ECDs from these two species might explain why LH specificity of eLH and eCG only exists in these species.

It has been reported that the GPA2/GPB5 heterodimer binds to the TSHR in mammals (6, 42, 109) but the receptors for GPA2/GPB5 or GPA2 and GPB5 independently in invertebrates are obviously not TSHR but are suspected to be GPHR ancestors and thus to belong to the GPCR family exhibiting a LRRD. In *Drosophila melanogaster*, the DLGR1 GPCR with LRRD has been reported to bind human GPA2/GPB5 (43). The LGR1 receptor gene has also been identified in the mosquito *Aedes aegypti* where this receptor has been proposed to regulate ion-transport across the hindgut (47). In *Caenorhabditis elegans*, only one GPCR gene with LRRD has been identified (*fsH-1*) that exhibits the highest similarity with FSHR among mammalian GPHR and plays a role in innate immune response (110). In this species, genes apparented to GPA2 (*flr-2*) and GPB5 (*flr-5*) were identified and might regulate FSH-R1 in the neural control of intestinal functions

(49), maybe including intestinal ion-transport and/or intestinal immune defense.

TRANSMEMBRANE SIGNAL TRANSDUCTION

The transmembrane (TM) signaling by GPHRs is due to the transconformation of their 7-TMD upon hormone binding to their ECD and subsequent interaction with the extracellular loops and/or extracellular amino-acids of their 7-helical TM sequences.

Precious structural information has been gained from the crystallographic studies of hormone–receptor ECD complexes mentioned above. No such information is available concerning the whole receptor and thus concerning ECD/7-TMD interaction upon GPH binding.

It had been shown a long time ago that LH is able to activate its receptor deprived of its ECD but with a low K_d (10^{-7} M instead of 10^{-10} M approximately) (111). This result suggests that the hormone ligand itself can directly contact the 7-TMD albeit with low affinity and promote its activating transconformation. The ECD might thus “catch” the hormone at very low physiological concentration and greatly favor its interaction with the 7-TMD so that transconformation of the latter can occur. In this first model, the hormone would thus be sandwiched between the ECD and the 7-TMD.

Determination of the 3D structure of GPCR 7-TMD has succeeded in only a limited number of them giving a static view yet they are expected to be highly dynamic and to assume different conformational states. These different conformations are functionally important as they are stabilized through the binding of the receptor with agonists or antagonists, with another receptor (dimerization) or with downstream partners (proteins G or others). These trans-conformations between inactive and active states affect mainly the relative orientations and distance of the seven TM sequences that can be detected intracellularly by the downstream partners (112). Repulsive separation of TM3 and TM6 in the TSHR 7-TMD is linked to receptor constitutive activation (113).

Glycoprotein hormone receptors are GPCRs and as such convey their signaling information mainly through interaction with heterotrimeric G-proteins mainly Gs but also Gi or Gq. Upon stimulation by their cognate GPH, GPHRs interact with the α -subunit in Gs protein and promote the exchange of GDP for GTP in this α -subunit. The α_s -GTP subunit then separates from the $\beta\gamma$ subunits complex and interacts with the adenylate cyclase to increase its activity and thus intracellular cyclic AMP concentration (114). This very general mechanism will not be detailed here.

INTRACELLULAR SIGNALING DOWNSTREAM TO GPHRs

The main partner GPHRs is the heterotrimeric Gs protein that stimulates membrane adenylate cyclase activity and consecutively leads to intracellular increase in cyclic AMP and thus to protein kinase A (PKA) stimulation. Specific phosphorylation of numerous proteins on threonine and/or serine residues is catalyzed by PKA. Among these proteins, there are transcription factors and various metabolic enzymes and structure proteins so that the biological responses to GPHs are genomic and/or metabolic and/or morphological. Thorough description of these cellular responses would be interesting but outside the scope of the present chapter.

We focus here on such issues only for examples related to the structure of GPHs or GPHRs.

After their phosphorylation by GRK, GPHRs recruit arrestin that promotes desensitization of the Gs pathway. In addition, arrestin acts as a scaffolding protein and recruits MAPKKK (Raf), MAPKK (MEK), and MAPK (ERK) and stimulates this pathway. It is thus interesting to note that arrestin do not act solely for arresting Gs signaling but also to initiate another signaling pathway (115). Therefore, arrestin is not only an interruptor but a commutator in signaling pathways downstream of GPHRs stimulation.

GPGR DESENSITIZATION AND INTERNALIZATION

Upon stimulation of GPHR by their cognate GPH, activated α_s -GTP of the Gs protein separates from the $\beta\gamma$ subunits complex. This complex recruits a GRK (GPCR kinase) that can in turn phosphorylate the activated GPHR at one or several specific locations in its intracellular sequences. These phosphorylated residues are target sites for arrestin that then interferes with the receptor–Gs protein interaction and thus desensitizes this pathway. In addition, as indicated before, arrestin also acts as a scaffolding protein that recruits MAPKKK, MAPKK, and MAPK thus initiating the stimulation of this pathway. Downstream the LH-induced LHR activation, it is the arrestin-3 isoform that is involved in the MAPK cascade activation in MA-10 cells (116).

Upon further hormone stimulation, receptor internalization occurs in addition to desensitization. This step is promoted by the arrestin-induced clathrin-coated pits that engulf GPHRs intracellularly into the endosomes. In the case of the TSHR, it is the arrestin-2 isoform that promotes internalization through this pathway (117). The fate of the internalized receptors is either degradation or recycling back to the plasma membrane in various proportions. The monomeric G-proteins Rab are responsible for this trafficking by promoting vesicle budding and fusion. These Rab proteins are GTPases like the Gs α -subunit: they are activated by exchanging GDP for GTP under the action of a guanine nucleotide exchange factor (GEF) and they are inactivated by hydrolyzing this GTP back to GDP.

Their GTPase activity permits their auto-inactivation which is enhanced by GTPase activating proteins (GAPs). Among the 60 Rabs known so far, Rab5a which is located to early endosome, mediates GPCR internalization. Indeed, Rab5a facilitates LHR internalization but it also favors its degradation and inhibits its recycling (118).

PHYSIOPATHOLOGICAL CONDITIONS AND CLINICAL CONSEQUENCES

The most numerous diseases involving gonadotropins are due to diminished (congenital hypogonadotropic hypogonadism) or excessive (adenomas) levels of secretion. The first are due to fetal defect in GnRH neuron migration, or a defect of pituitary development or from a functional defect of the hypothalamic–pituitary axis between GnRH neurons and gonadotrope cells (Kallmann syndrome). The latter arises following the development of LH-, FSH-, or TSH-secreting pituitary adenomas (119). The study of these conditions is beyond the scope of the present paper as they are not related to the structure of gonadotropins or of their receptors.

Natural mutations in GPH genes and in their receptors' genes are either activating (gain of function) or inhibiting (loss of function). The former are expressed as a dominant trait, thus in the heterozygous state, whereas the latter are only expressed when biallelic. In addition, some mutations affect internalization and/or degradation of GPHRs.

PATHOLOGICAL CONDITIONS DUE TO HORMONE STRUCTURE MODIFICATIONS

Pathological conditions due to mutations in gonadotropin or thyrotropin gene coding sequences and hence to modifications in their polypeptide structure are fairly rare. However, some pathological conditions are due to or are related with modifications in the saccharide side chains.

For example, trophoblastic cells from trisomy 21 pregnancy produce hyper-glycosylated forms of hCG with low biological activity (120). Detection of hCG variants has also been shown to be related to various malignancies in human (121).

Abnormal stimulation of TSHR by hCG during pregnancy has also been described. A small proportion of these patients have clinical hyperthyroidism, termed gestational thyrotoxicosis (102). They either secrete a variant of hCG with increased thyroid-stimulating activity or their TSHR has increased affinity for hCG. A unique family with recurrent gestational hyperthyroidism associated with hyperemesis gravidarum was found to have a mutation in the ECD of the TSHR that made it responsive to normal levels of hCG (122).

While mutations of gonadotropin and TSH genes in human are exceedingly rare, genetic alterations of their respective receptors are more frequent. These mutations can lead either to constitutive activation of the receptors or to their inactivation, i.e., their inability to respond to their cognate hormone for various reasons.

PATHOLOGICAL CONDITIONS DUE TO FSHR

Inactivating mutations

Inactivating mutations of FSHR gene, in women, are generally associated with primary ovarian insufficiency. Numerous inactivating FSHR mutations have been described such as Ala189Val in the FSHR ECD leading to hypergonadotropic hypogonadism with no or weak response to FSH (123, 124). A Pro519Thr mutation in the second intracellular loop of the 7-TMD was associated to elevated serum FSH concentration, low estrogen and inhibin concentrations, and hypoplastic uterus and ovaries. The inactivity of the receptor was found not to be due to defect in its signal transduction ability but to its intracellular trapping, and therefore to its absence at the cell surface. In patients with such total loss of FSHR function, there is no passage from primary to secondary follicles (125) and this block of course causes infertility.

Activating mutations

In contrast to the numerous inactivating mutations in FSHR, only one example of activating mutation has been described in human: Asp567Gly in the third intracytoplasmic loop (126). The D550G mutation in the FSHR 7-TMD has been recently shown to uncouple the link between internalization and degradation of hFSH. It is therefore expected to be more efficiently recycled.

Extragonadal expression

In addition to FSHR structure modifications due to mutations, diverse pathologies are a consequence of its anomalous extragonadal expression. FSHR is normally expressed in extragonadal reproductive tissues such as placenta (127) but also in the endothelial cells associated with a diverse range of solid tumors (128). Genitourinary malignancies were strongly represented (prostate adenocarcinomas, urothelial carcinomas, renal cell carcinomas, and seminomas). The ubiquitous nature of FSHR in tumor blood neovasculature suggests a biological role in human solid tumors possibly through induction of vascular endothelial growth factor (VEGF) in granulosa cells. FSHR has also been shown to be expressed in normal prostate tissue but at significantly lower levels than in prostate cancer.

The presence of the Ser680 FSHR isoform in Taiwanese women was found to be associated to a lower occurrence of endometriosis (129). This suggests the presence of functional FSHR in invading endometrial cells.

PATHOLOGICAL CONDITIONS DUE TO LHR STRUCTURE MODIFICATIONS

Activating and inactivating mutations in LHR with very different phenotypic effects have been identified. Inactivating mutations in the LHR are responsible for male pseudohermaphroditism or Leydig cell hypoplasia in individuals with 46 XY karyotypes, characterized by a predominantly female phenotype. Activating mutations in the LHR are responsible for precocious puberty due to its constitutive activity in the absence of hormone.

Inactivating mutations

Inactivating mutations of LHR gene most often affect XX individuals whose families also include cases of male pseudohermaphroditism. Clinically, these women suffer from primary amenorrhea but with normal development of breast and pubic hair.

Naturally occurring LHR mutant without the polypeptide sequence encoded by exon 8 causes Leydig cell hypoplasia due to the loss of hormone-binding ability. The LHR mutant lacking exon 9 was found not to be addressed to the plasma membrane thus also leading to insensitivity to the hormone. Recently, a novel cryptic exon (exon 6A) was found in LHR gene (130) that leads to the synthesis of an incomplete mRNA variant encompassing exons 1–6–6A (6A terminal variant). A mutation in exon 6A (A557C) leads to an overexpression of this shortened mRNA as well as that of an mRNA including exons 1–6–6A–7–11 (6A internal variant) in Leydig cells leading to an insufficient amount of full-length mature LHR at the cell surface. Like inactivating mutations in the LHR coding sequence, the overexpression of LHR mRNA including the 6A exon is thus responsible for male pseudohermaphroditism or Leydig cell hypoplasia (130). These three examples among many others indicate that the insensitivity of LHR to its cognate hormones LH and HCG may have very different causes (binding deficiency, intracellular trapping, or transcription inhibition).

Activating mutations

Activating mutations in the LHR gene are one of the most common mutations found in the GTHR genes. These mutations promote precocious puberty in boys but no obvious phenotype in females (131).

There are no reports of naturally occurring activating mutations in the ECD although, engineered mutations of a serine residue in the hinge region of LHR result in constitutive activity of receptors expressed in recipient cells (132). One of the most common activating mutations is the missense mutation D578G in TM6 of the TMD. Another mutation at the same position (D578H) was found to be highly activating. To date, there is no report of women with a D578H mutation; this mutation has appeared only as a somatic mutation restricted to Leydig cell tumors in boys ranging in age from 5 to 8 years, suggesting that this particular mutation is incompatible with germ-line transmission (131).

Another constitutively activating mutation in the TM3 of the hLHR (L457R) has been identified in only one boy with gonadotropin-independent precocious puberty (133). Interestingly, the amino-acid in this location forms a salt bridge with the amino-acid in position 578 that is also prone to activating mutations as described above. The L457R mutation in LHR has also been found to diminish lysosomal degradation of the receptor and this could also contribute to its constitutive activity by prolonging the duration of signaling (134).

The N312S mutation of LHR does not lead to any functional effect but has been shown to be moderately but significantly related to increased breast cancer (135). The reason of this link is unknown.

PATHOLOGICAL CONDITIONS DUE TO TSHR

Activating mutations

Activating mutations of the TSHR are rare. Nevertheless, an heterozygous substitution in exon 10 (Ile568Thr) leads to neonatal thyrotoxicosis without anti-TSHR antibodies production (136). Likewise, a Leu665Phe mutation in TSHR TM helix 7 leads to non-autoimmune hyperthyroidosis (137).

Inactivating mutations

Inactivating mutations in human TSHR also exist in a few occurrences. For example, the Gln489His mutation in the first extracellular loop, leads to hypothyroidism (138).

TSHR autoantibodies

Graves' disease (elevated thyroid hormone levels and low to undetectable TSH) is a leading cause of hyperthyroidism worldwide. It arises from the action of TSHR stimulating autoantibodies. TSHR autoantibodies are either stimulating (cAMP/PKA/CREB and/or AKT/mTOR/S6K signaling cascades) or inhibiting TSH effects, or neutral autoantibodies that induce thyroid cell apoptosis via reactive oxygen species (ROS) generation.

In contrast to the gonadotropin receptors FSHR and LHR, TSHR ECD is matured by proteolysis and is connected to the TM domain through a disulfide bridge (139). The unshed TSHRs or disulfide cleaved soluble ECD forms and/or TM forms might be much more immunogenic than the functional matured receptors with SS-bridged subunits.

GPA2/GPB5 binding

Since GPA2/GPB5 heterodimer has been proposed to bind to TSHR in vertebrates, it is interesting to point out that GPB5^{-/-} mutant mice exhibit transient hypothyroxinemia (109) whereas

mice overexpressing GPB5 show a resistance to diet-induced obesity (45). The involvement of defects in GPA2, GPB5, or TSHR should thus eventually be taken in consideration in thyroid axis pathologies.

CONCLUSION AND PERSPECTIVES

Because of their implication in important physiological functions and their structural complexity, GPHs and their receptors remain a difficult and active field of investigation. The complexity of their structure makes the pharmaceutical production and control of recombinant hormones a heavy task. For example, hFSH with a fused hCG β CTP (Figure 3) which is now on the market (corifollitropin; Elonva®) in an increasing number of countries clearly exhibits a longer half-life that permits to diminish the number of injections to the patients. Nevertheless, there is a possible risk of ovarian hyperstimulation in some of them.

Since GPCRs are the main targets of pharmaceuticals, it can be envisioned that synthetic small-molecular weight drugs will be, sooner or later, available to finely tune the activation of LHR and FSHR for fertility treatments. A number of such molecules have already been synthesized and tested (140–144) and structure modeling (145, 146) is expected to be particularly helpful in the molecular design of new drugs of this type.

Concerning their ancestors, GPA2 and GPB5, their putative heterodimerization is still a matter of debate as well as their biological activities either on their own or after combination.

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Pineal melatonin is a circadian time-giver for leptin rhythm in Syrian hamsters

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Nocturnal secretion of melatonin from the pineal gland may affect central and peripheral timing, in addition to its well-known involvement in the control of seasonal physiology. The Syrian hamster is a photoperiodic species, which displays gonadal atrophy and increased adiposity when adapted to short (winter-like) photoperiods. Here we investigated whether pineal melatonin secreted at night can impact daily rhythmicity of metabolic hormones and glucose in that seasonal species. For that purpose, daily variations of plasma leptin, cortisol, insulin and glucose were analyzed in pinealectomized hamsters, as compared to sham-operated controls kept under very long (16 h light/08 h dark) or short photoperiods (08 h light/16 h dark). Daily rhythms of leptin under both long and short photoperiods were blunted by pinealectomy. Furthermore, the phase of cortisol rhythm under a short photoperiod was advanced by 5.6 h after pinealectomy. Neither plasma insulin, nor blood glucose displays robust daily rhythmicity, even in sham-operated hamsters. Pinealectomy, however, totally reversed the decreased levels of insulin under short days and the photoperiodic variations in mean levels of blood glucose (i.e., reduction and increase in long and short days, respectively). Together, these findings in *Syrian hamsters* show that circulating melatonin at night drives the daily rhythmicity of plasma leptin, participates in the phase control of cortisol rhythm and modulates glucose homeostasis according to photoperiod-dependent metabolic state.

Keywords: golden hamster, pinealectomy, photoperiod, cortisol rhythm, plasma glucose

Introduction

Photoperiodic animals, such as *Syrian hamsters*, are species whose physiology is specifically and reversibly regulated on a seasonal basis. Notably, they display seasonal changes in gonadal activity. When *Syrian hamsters* are exposed to long, summer-like photoperiods, gonadal function is active and their adiposity is decreased. Conversely, after transfer to short, winter-like photoperiods, these hamsters become sexually quiescent, while their adiposity increases (Bartness and Wade, 1985).

In mammals, melatonin can be synthesized by several organs, including the pineal gland, retina and gastrointestinal tract (Pévet, 2003; Hardeland et al., 2011; Tosini et al., 2012). The pineal gland, however, is the main source of the nocturnal peak of circulating melatonin, as evidenced by the lack of detectable levels in the blood after pinealectomy. The daily fluctuations of melatonin synthesis and release by the pineal are tightly controlled by the master clock in the suprachiasmatic nuclei of

the hypothalamus (SCN). The daily duration of nocturnal melatonin transduces photoperiodic cues into neuroendocrine changes that modulate seasonal physiology, thus highlighting the pivotal role of pineal melatonin in the integration of seasonal changes in day length (Malpoux et al., 2001; Pevet, 2003).

Besides, the nocturnal peak of melatonin may also play a role as an internal time-giver on a daily basis (Pevet and Challet, 2011). Among others, circadian rhythmicity of the pups during gestation and weaning can be synchronized by maternal melatonin via placenta and milk (Torres-Farfan et al., 2008, 2011). In adults, nocturnal melatonin can control the rhythmic activity of brain and peripheral regions, including striatum, pars tuberalis of the hypophysis and spleen, as shown by the disappearance of clock gene oscillations in these structures after pinealectomy (Messager et al., 2001; Uz et al., 2003; Prendergast et al., 2013). Melatonergic cues may even feed-back on the master clock in the SCN where they affect clock gene expression (Agez et al., 2007) and firing rate (Rusak and Yu, 1993).

The aim of the present study was to investigate whether rhythmic melatonin can also affect the daily timing of other hormones, such as leptin and glucocorticoids. Plasma leptin is secreted by adipocytes in proportion with adiposity (Ahima and Flier, 2000). Accordingly, levels of plasma leptin are higher in *Syrian hamsters* adapted to short photoperiod compared to animals exposed to long photoperiod (Horton et al., 2000). In addition, leptin is rhythmically secreted, with peak phases depending on the species. Adrenal glucocorticoids are other hormones rhythmically secreted, with endogenous peaks occurring around activity onsets (Dickmeis, 2009). The main glucocorticoid is cortisol in humans and hamsters, and corticosterone in rats and mice. To test whether nocturnal melatonin can have chronomodulatory effects on leptin, cortisol and insulin rhythms, we investigated the impact of pinealectomy on these rhythms in *Syrian hamsters* kept under long or short photoperiods.

Material and Methods

Animals

Ninety-seven male *Syrian hamsters* (*Mesocricetus auratus*) bred in-house (Chronobiotron platform, UMS3415, CNRS and University of Strasbourg) were 6-month-old at the end of the experiment. From birth, they were maintained in a Long Photoperiod (LP) consisting of 16-h light and 8-h dark (around 150 lux within the cages during the light period), with lights on at 05:00 AM, defining Zeitgeber time (ZT) 0. Animals were housed 3–5 per cage and kept at $22 \pm 1^\circ\text{C}$ with *ad libitum* access to water and food. All experiments were conducted in accordance with the French National Law (License 67–88) implementing the European Union Directive 2010/63/EU. All efforts were made to minimize the number of animals used and their suffering, and the study met the ethical standards.

Experimental Design

All hamsters were initially kept under LP. Around half of the animals were sham-operated ($n = 48$), while the others were pinealectomized ($n = 49$).

Hamsters (weighing approximately 140 g) were anesthetized during the light phase with i.p. injections of a mixture of Zoletil 20 (Virbac, Carros, France) and Rompun (Bayer Pharma, Puteaux, France). After being placed into a stereotaxic instrument (Kopf), a midline circular incision of the skull was gently performed to expose the pineal gland. The pineal was removed with a pair of fine forceps, the skull cap replaced and the incision closed. After surgery, hamsters were housed individually for 1–2 days until complete recovery. Thereafter, hamsters were either kept in LP ($n = 21$) or transferred to a Short Photoperiod (SP; $n = 28$) consisting of 8 h light and 16 h dark, with lights on at 09:00 AM, defining ZT0 under SP. This photoperiodic condition triggers in that species an inhibition of the reproductive axis, including gonadal atrophy.

Ten weeks later, all animals under LP and SP were killed every 4 h ($n = 1$ of the same cage per ZT) throughout the 24 h, from ZT3, 7, 11, 15, 19 to 23.

Blood samples were collected in heparinized tubes and centrifuged for 10 min at 4600 g at 4°C .

Hormonal Assays

Plasma concentrations of leptin were determined by a multi-species leptin RIA kit (XL-85K, Millipore, Molsheim, France). The limit of sensitivity of the assay was $2\text{ ng}\cdot\text{mL}^{-1}$ and the inter- and intra-assay coefficients of variance were <9 and $<4\%$, respectively.

Plasma concentrations of cortisol were determined by a Cortisol Express EIA Kit (AYN830, Cayman Chemical, Ann Arbor, MI, USA). The limit of sensitivity of the assay was $0.1\text{ ng}\cdot\text{mL}^{-1}$, while the coefficients of variance for a dose of 5 ng were equal, respectively, to 11 and 6% for inter- and intra-assays. Plasma levels of insulin were assayed with Ultra Rat Insulin ELISA Kit (# 90060, Crystal Chem, Downers Grove, IL, USA) using hamster insulin standard (# 90330, Crystal Chem) following the instructions of the manufacturer. The limit of sensitivity of the assay was $0.1\text{ ng}\cdot\text{mL}^{-1}$ and the coefficients of variance were $<10\%$ for both inter- and intra-assays.

Glucose Assay

Blood glucose was determined with GOD-PAP Kit (LP80009, Biolabo, Maizy, France).

Statistical Analysis

Data are presented as means \pm S.E.M. Three-Way analyses of variance (ANOVA) followed by *post-hoc* comparisons with the Fisher's least significant difference Test were used to compare the effects of Zeitgeber time \times photoperiod \times treatment. Level of significance was set at $P < 0.05$. For each blood parameter in either photoperiod, One-Way ANOVAs, or ANOVAs on ranks when normality test (Shapiro–Wilk) failed, were performed to determine if the effect of Zeitgeber time was significant (i.e., if the blood parameters display time-dependent variations). For assessing daily rhythmicity, we used a cosinor analysis to determine mean level, amplitude and acrophase of the considered parameter with SigmaPlot software (Systat software Inc., San Jose, CA, USA). Individual data of each experimental group (Long or short photoperiod, sham-operated

or pinealectomized) were fitted to the following regression: $[y = a + b \cdot \cos(2 \cdot \pi \cdot (x - c) / 24)]$ where a is the mean level, b the amplitude, and c the acrophase of the rhythm. For a given parameter, we used the Mean, Size, standard Error (MSE) format of SigmaPlot to compare the acrophases of significant regressions with t -tests or One-Way ANOVA followed by Fisher's *post-hoc* test for two or more groups, respectively.

Results

Body mass at the end of the experiment was not significantly modified by pinealectomy, either in LP or SP (**Table 1**). Pinealectomy is routinely used in this and other labs to suppress the nocturnal rise of circulating melatonin. Indeed, after visual extirpation of the pineal gland in hamsters, levels of plasma melatonin at night become systematically undetectable (i.e., less than 1 pg/tube according to the limit of sensitivity of our RIA melatonin assay) (Schuster et al., 2001). In this study, we attribute the differences in pinealectomized hamsters compared to sham-operated controls, as due to impaired melatonin secretion. Based on the present protocol without hormonal replacement in pinealectomized animals, we cannot fully exclude the possibility of melatonin-independent effects of pinealectomy.

Cortisol Rhythm

Levels of plasma cortisol were significantly affected by Zeitgeber time (Three-Way ANOVA; $P = 0.01$). Independently of photoperiod and treatment, higher values were found at ZT11 (i.e., 11 h after lights on) compared to ZT3 (early morning) and ZT23 (late night; **Figure 1**). There was also a significant effect of photoperiod (Three-Way ANOVA; $P = 0.003$) on plasma cortisol, mean values being larger under short days compared to long days. By contrast, plasma cortisol was not modified by Treatment (Three-Way ANOVA; sham-operated vs. pinealectomized; $P = 0.09$). In addition, the triple interaction [Zeitgeber time \times photoperiod \times treatment] was significant (Three-Way ANOVA; $P = 0.004$), but only for sham-operated animals, indicating that in these animals the daily pattern differs between photoperiods. Cosinor analysis detected a significant daily rhythm for both sham-operated and pinealectomized hamsters under short photoperiod (**Table 2**). Of note, the cortisol acrophase was phase-advanced by almost 6 h in pinealectomized hamsters compared to intact animals (from ZT14.3 to ZT8.7; $P < 0.05$; One-Way ANOVA; **Figure 1**; **Table 2**). By contrast, under long photoperiod, a significant daily rhythm was found only in pinealectomized hamsters (delayed by more than 5 h as compared to pinealectomized hamsters under short days; $P <$

0.05; One-Way ANOVA; **Table 2**). The lack of significant daily rhythmicity in intact animals under long days is probably due to the bimodal pattern characterized by daytime and nocturnal peaks (**Figure 1**).

Leptin Rhythm

Levels of plasma leptin were changed according to Zeitgeber times (Three-Way ANOVA; $P = 0.002$), the values at ZT15 being larger than those at both ZT11 and ZT23 (**Figure 2**). Moreover, the levels of leptin were modified by photoperiod (Three-Way ANOVA; $P = 0.004$), with increased values in short days. Levels of plasma leptin were not affected by pinealectomy (Three-Way ANOVA; $P = 0.9$). However, the triple interaction [Zeitgeber time \times photoperiod \times treatment] was significant (Three-Way ANOVA; $P = 0.003$), but only after pinealectomy, indicating that the daily patterns of leptin in these animals differ in both photoperiods, as compared to respective controls (**Figure 2**). Accordingly, a significant rhythm was detected only in sham-operated animals, demonstrating a role of time-giver of circulating melatonin upon leptin rhythm (**Figure 2**; **Table 2**). Furthermore, the leptin acrophase under long photoperiod was delayed by 8 h as compared to short photoperiod (from ZT13.2 to ZT21.3; $P < 0.05$; t -test; **Table 2**).

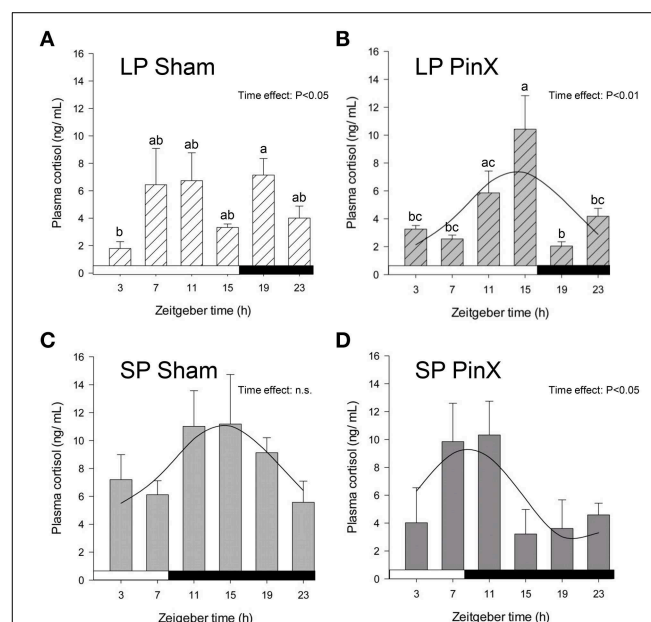


FIGURE 1 | Effects of photoperiod and pinealectomy on daily variations of plasma cortisol in the Syrian hamster. Light and dark bars represent photoperiod and scotoperiod, respectively. LP, long photoperiod (16 h light/08 h dark) (**A,B**); SP, short photoperiod (08 h light/16 h dark) (**C,D**); Sham, sham-operated hamsters (**A,C**); PinX, pinealectomized hamsters (**B,D**). Fitted curves represent significant cosinor analyses (for details, see Materials and Methods and **Table 2**). The absence of a curve in the LP Sham panel indicates that the daily variations of cortisol are not significant in these conditions. The inset for each graph indicates the effect of Zeitgeber time, evaluated by One-Way ANOVAs or ANOVAs on ranks when normality test (Shapiro-Wilk) failed; n.s., non-significant. For a given panel, histograms lacking common letters are significantly different ($P < 0.05$).

TABLE 1 | Body mass of hamsters.

| | LP Sham (n = 23) | LP PinX (n = 21) | SP Sham (n = 25) | SP PinX (n = 28) |
|----------|---------------------|---------------------|---------------------|---------------------|
| Mean (g) | 129.7 | 127.6 | 136.5 | 131.9 |
| ±SEM | ±2.9 | ±2.6 | ±2.7 | ±2.1 |

Mean body mass of hamsters, either sham-operated (Sham) or pinealectomized (PinX) under long (LP) or short photoperiods (SP).

TABLE 2 | Parameters of cosinor regressions.

| | | Plasma leptin | | | Plasma cortisol | | | Plasma insulin | | | Plasma glucose | | |
|--------------------------|---|--------------------|------|--------|-------------------|------|--------|--------------------|------|--------|----------------|-------|--------|
| | | Mean | SEM | P | Mean | SEM | P | Mean | SEM | P | Mean | SEM | P |
| LP Sham (<i>n</i> = 23) | a | 7.06 | 0.49 | <0.001 | 5.03 | 0.71 | <0.001 | 1.79 | 0.22 | <0.001 | 1.02 | 0.05 | <0.001 |
| | b | 2.18 | 0.70 | 0.006 | 1.46 | 1.02 | 0.17 | 0.21 | 0.32 | 0.53 | 0.04 | 0.08 | 0.62 |
| | c | 21.34* | 1.22 | <0.001 | 13.43 | 2.60 | <0.001 | 6.68 | 5.36 | 0.27 | 14.60 | 7.20 | <0.001 |
| LP PinX (<i>n</i> = 21) | a | 6.95 | 0.67 | <0.001 | 4.72 | 0.65 | <0.001 | 1.78 | 0.10 | <0.001 | 1.24 | 0.03 | <0.001 |
| | b | 0.75 | 0.95 | 0.43 | 2.66 | 0.92 | 0.009 | 0.38 | 0.15 | 0.017 | 0.05 | 0.04 | 0.19 |
| | c | 6.75 | 4.80 | 0.17 | 14.08* | 1.32 | <0.001 | 4.84* | 1.48 | 0.004 | 13.84 | 2.81 | <0.001 |
| SP Sham (<i>n</i> = 25) | a | 8.48 | 0.61 | <0.001 | 8.27 | 0.74 | <0.001 | 1.32 | 0.09 | <0.001 | 1.12 | 0.05 | <0.001 |
| | b | 2.45 | 0.85 | 0.01 | 2.82 | 1.09 | 0.015 | 0.12 | 0.13 | 0.37 | 0.11 | 0.06 | 0.09 |
| | c | 13.22 [#] | 1.34 | <0.001 | 14.26* | 1.36 | <0.001 | 18.92 | 4.22 | 0.11 | 18.80 | 2.36 | <0.001 |
| SP PinX (<i>n</i> = 28) | a | 8.70 | 0.05 | <0.001 | 6.01 | 0.88 | <0.001 | 1.81 | 0.20 | <0.001 | 1.00 | 0.035 | <0.001 |
| | b | 0.11 | 0.06 | 0.91 | 3.30 | 1.21 | 0.011 | 0.61 | 0.28 | 0.037 | 0.10 | 0.05 | 0.041 |
| | c | 8.81 | 2.36 | 0.81 | 8.67 [#] | 1.45 | <0.001 | 20.18 [#] | 1.79 | <0.001 | 12.96 | 1.81 | <0.001 |

The table shows the three parameters of cosinor regression, including a the mean level, b the amplitude, and c the acrophase of the rhythm (see Material and Methods for details). For the acrophase, the reference time is Zeitgeber 0 (i.e., lights on) for both photoperiods. LP, long photoperiod; SP, short photoperiod; Sham, sham-operated; PinX, pinealectomized. *Italicized parameters are not statistically significant* ($P > 0.05$). For a given hormone (column), mean acrophases lacking common superscripts (* or #) are significantly different (t-test or One-Way ANOVA for 2 or more groups, respectively). No superscript is included in case the overall regression was not significant due to non-significant acrophase (c) and/or amplitude (b), or when there was only one significant regression precluding statistical comparison with the other groups (as in the case of plasma glucose).

Insulin Rhythm

The main effects of Zeitgeber time, photoperiod and treatment were not significant for insulin levels (Three-Way ANOVA; $P = 0.14$, $P = 0.17$, and $P = 0.11$, respectively). However, the double interactions [Zeitgeber time \times photoperiod] and [Zeitgeber time \times treatment] were significant (Three-Way ANOVA; $P = 0.01$ for both), indicating that the daily profiles of insulin differ according to photoperiodic conditions. In particular, the mean levels of plasma insulin were lower in hamsters exposed to short photoperiod as compared to long photoperiod, but only for sham-operated animals (Fisher's *post-hoc* test; $P = 0.02$). Finally, the double interaction [photoperiod \times treatment] was also significant (Three-Way ANOVA; $P = 0.04$), revealing that insulin levels were increased by pinealectomy under short photoperiod (Fisher's *post-hoc* test; $P < 0.01$), but they were unaffected by this treatment under long photoperiod (**Figure 3; Table 2**). Cosinor analysis detected a significant daily rhythm of plasma insulin only in pinealectomized hamsters, with different acrophases between short and long photoperiods (**Figure 3; Table 2**).

Glucose Rhythm

Levels of blood glucose were hardly affected by Zeitgeber time (Three-Way ANOVA; $P = 0.056$), and unchanged by photoperiod (Three-Way ANOVA; $P = 0.4$) or treatment (Three-Way ANOVA; $P = 0.6$). The lack of daily rhythmicity in blood glucose might be due to the 4-h sampling. Nonetheless, the double interaction [Zeitgeber time \times treatment] was highly significant (Three-Way ANOVA; $P < 0.001$). In particular, mean blood glucose in pinealectomized hamsters was significantly increased under long photoperiod, while it was decreased in short photoperiod (Fisher's *post-hoc* test; $P < 0.001$, **Figure 4**;

Table 2). Moreover, mean glycemia in sham-operated hamsters was increased in short vs. long photoperiods (Fisher's *post-hoc* test; $P = 0.007$), while the values in pinealectomized animals was decreased in short vs. long photoperiods (Fisher's *post-hoc* test; $P < 0.001$). Cosinor analysis detected a significant daily rhythm of blood glucose only in pinealectomized hamsters under short days (**Figure 4; Table 2**), precluding any comparison with the other studied groups. Nevertheless, these results indicate that circulating melatonin differentially affects overall glucose regulation according to the photoperiod-induced metabolic changes.

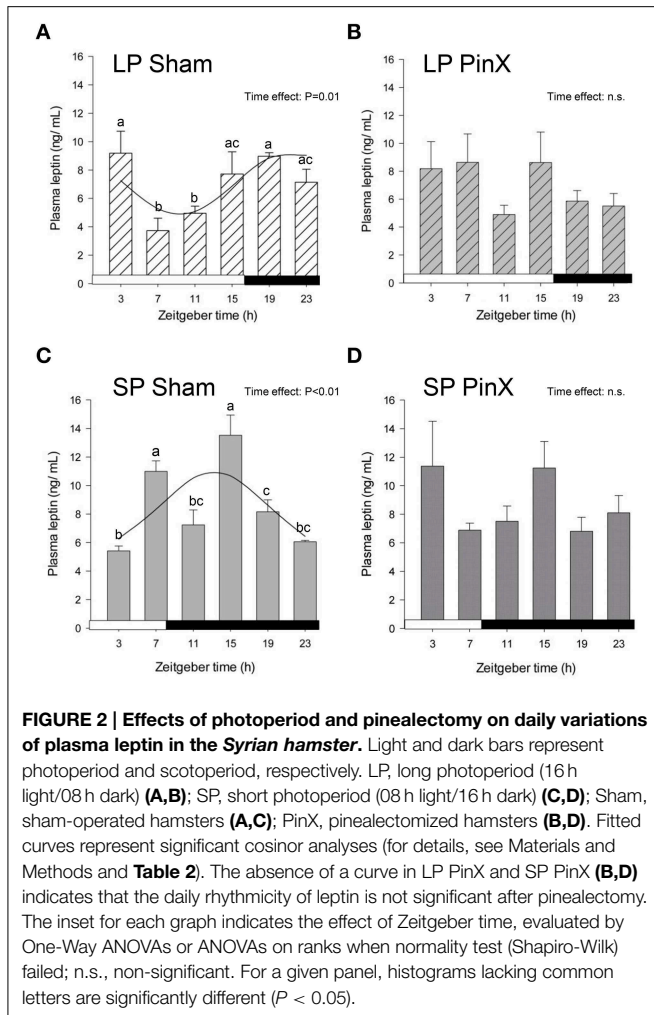
Discussion

The main findings of this study are that pineal melatonin not only acts as a time-giver for hormonal rhythms, but also affects glucose homeostasis in a photoperiod-dependent way.

The first conclusion is supported by the fact that pinealectomy in *Syrian hamsters* suppresses the daily rhythmicity of plasma leptin and can lead to shifts of the daily rhythm of plasma cortisol. The second conclusion relies on the differential impact of pinealectomy on glycemia and plasma insulin according to the photoperiod (**Figure 5**).

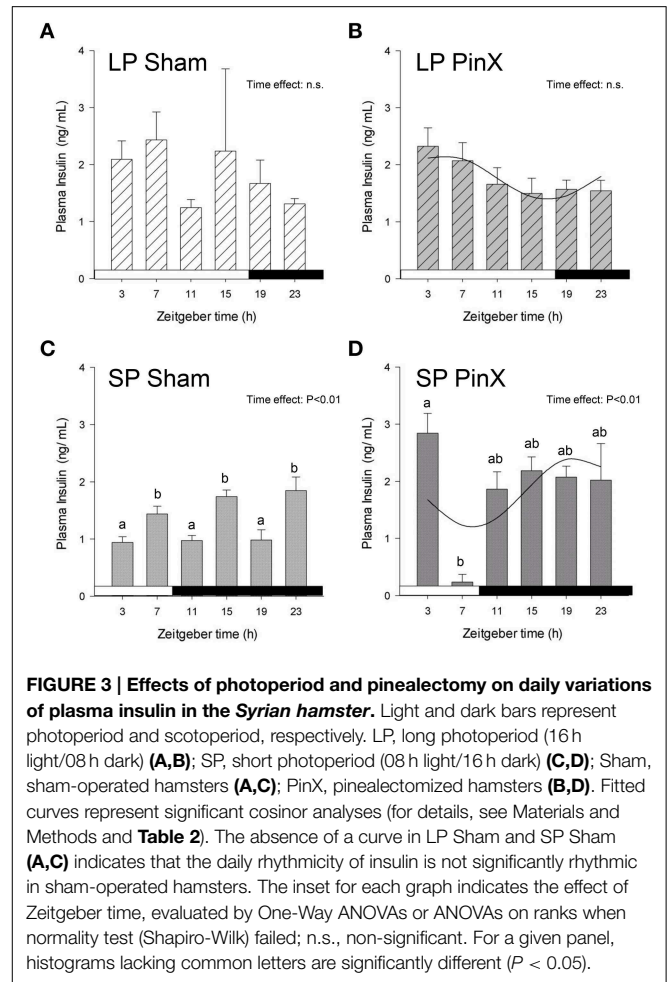
Pinealectomy Affects Glucose and Insulin Levels According to Photoperiods

This study is mainly based on cosinor analysis with a relatively small number of observations per time point ($n = 4-6$) and 4-h sampling (6 time-points over 24 h). In contrast to what is found in other rodents (La Fleur et al., 1999; Kalsbeek et al., 2001; Cuesta et al., 2009), no significant daily rhythm in blood glucose is found in control (sham-operated) *Syrian hamsters* housed under long



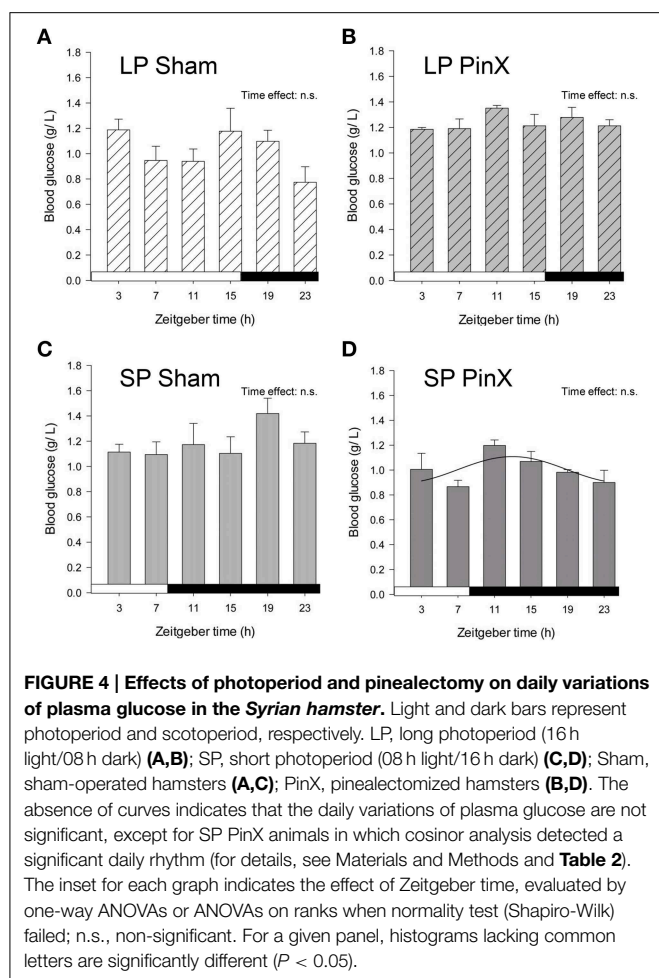
or short photoperiods. This prevented us to assess the possible time-giving property of melatonin on that parameter. Based on a study in pinealectomized rats, the endogenous rhythm of pineal melatonin may enhance the amplitude of the daily rhythm of plasma glucose (la Fleur et al., 2001). In any case, the lack of rhythmic glycemia in Syrian hamsters has been already reported (Rowland, 1984). This specific feature is actually concomitant with a lack of marked day-night difference in feeding behavior (Rowland, 1984). Because hamsters in the present study were not fasted before blood sampling, meal-induced rise in plasma glucose may have blunted any small endogenous rhythm.

Contrary to mice fed *ad libitum* (Ahren, 2000), no daily variations of plasma insulin were found in intact Syrian hamsters exposed to either long or short photoperiods. Nonetheless, a significant rhythm of plasma insulin with low amplitude was only detected in pinealectomized hamsters. As noted for glucose levels, possible interactions with meal-induced secretion of insulin may have interfered with the endogenous rhythmicity of insulin. Alternatively, the fed state of the hamsters studied here avoided fasting-induced bias in hormonal/glucose levels due to variable mobilization of body stores according to photoperiods, times of day and associated differences in fasting duration.



Nevertheless, the present study provides novel information on how melatonin can modulate glucose homeostasis. We found that the effects of pinealectomy on plasma glucose and insulin depend on the metabolic state (i.e., short-day, fatter phenotype with more plasma glucose and leptin, and less insulin vs. long-day, leaner phenotype with less plasma glucose and leptin, and more insulin). In relatively lean hamsters (exposed to long photoperiod), pinealectomy leads to mild hyperglycemia. This finding is in accordance with the fact that pineal ablation in non-photoperiodic lean rats also increases nighttime glucose levels (la Fleur et al., 2001). This relative hyperglycemia at night in rats without pineal gland is not due to a reduced insulin secretion to meal cues because in hamsters exposed to long photoperiod, mean insulin levels were not significantly affected by pinealectomy. Accordingly, another work in pinealectomized rats did not detect changes in plasma insulin levels and provides experimental arguments for a decreased sensitivity of cells to circulating insulin (Alonso-Vale et al., 2004). Therefore, the mild hyperglycemia in pinealectomized hamsters in long days may be, as observed in rats, due to a reduced responsiveness of the target cells to insulin.

In sharp contrast, pinealectomy in relatively fatty hamsters (exposed to short photoperiod) reduces the mild hyperglycemia



observed in sham-operated hamsters. Thus, pinealectomy in that case normalizes glycemia to levels close to those in long-day control hamsters. High-fat feeding triggers obesity in non-photoperiodic rats. Pineal ablation, however, does not modulate their concentration of blood glucose, at least in the morning (Prunet-Marcassus et al., 2003). In the present work, we also found that concentrations of plasma insulin were increased by pinealectomy in hamsters exposed to short photoperiod. Therefore, considering that insulin sensitivity is improved under short days compared to long days, the increased concentrations of circulating insulin can explain the lower levels of blood glucose. Meanwhile, our results reveal that the lack of circulating melatonin in hamsters markedly modifies glucose homeostasis, with opposite effects according to their seasonal metabolic state.

Pinealectomy Shifts the Daily Rhythm of Cortisol

In sham-operated hamsters, the daily pattern of plasma cortisol was found to be unimodal and bimodal in short and long photoperiods, respectively. Furthermore, plasma levels of cortisol are larger in short compared to long photoperiods. This contrasts with previous results in the Syrian hamster that found unchanged levels in males (de Souza and Meier, 1987) or decreased levels of cortisol in short days (Ottenweller et al., 1987; Nexon et al., 2011). These discrepancies are puzzling, and may be

related to the more extreme photoperiods used in the present study and the fact the hamsters here were all operated upon. Furthermore, the reported quiescent hypothalamo-hypophyso-adrenal axis in *Syrian hamsters* under short photoperiods can be profoundly modulated by social interactions (Morgan, 2012). It is therefore possible that the present housing conditions (3–5 animals per cage) may have led to social instability and/or inter-individual differences in social status, thus keeping an activated hypothalamo-hypophyso-adrenal axis in *Syrian hamsters* under short photoperiods. Regarding the effects of melatonin on glucocorticoids, pinealectomy in *Syrian hamsters* does not affect the mean levels of cortisol, whatever the photoperiod. This confirms what is found by several studies in rats for corticosterone levels (Szafarczyk et al., 1983; Kaplanski and Ronen, 1986), albeit other investigations report increased levels of plasma corticosterone in pinealectomized rats (Oxenkrug et al., 1984; Alonso-Vale et al., 2004).

In terms of daily timing, a clear phase-advance is induced by pinealectomy in hamsters exposed to short days. Previous studies in adult rats found no effect of pinealectomy on the phase of corticosterone profile (Szafarczyk et al., 1983; Kaplanski and Ronen, 1986), while adrenal glands of rat fetus is entrained by maternal melatonin (Torres-Farfan et al., 2011). Furthermore, melatonin modulates *in vitro* molecular oscillations in the adrenal gland of adult monkeys (Valenzuela et al., 2008). Together, these findings suggest that circulating melatonin can participate in the control of adrenal rhythmicity.

Pinealectomy Flattens the Daily Rhythm of Leptin

In accordance with the increased adiposity in hamsters exposed to short photoperiods (Bartness and Wade, 1985), mean levels of plasma leptin are higher in hamsters exposed to short photoperiods in comparison with animals in long photoperiods, as found by others (Horton et al., 2000). Furthermore, whatever the photoperiod, pinealectomy in hamsters does not affect mean leptin levels, in keeping with results in the non-photoperiodic laboratory rat (Alonso-Vale et al., 2004).

In the present study, the peak of plasma leptin in hamsters exposed to long or short photoperiods takes place at night, as in other nocturnal rodents like rats and mice (Ahren, 2000; Kalsbeek et al., 2001; Cuesta et al., 2009). Other reports in *Syrian hamsters*, however, found either hardly rhythmic profile (Horton et al., 2000) or rhythmic leptin peaking during daytime (Karakas and Gunduz, 2006). The daily rhythm of leptin is thought to be controlled both by the master clock in the SCN and by the adipose clock (Kalsbeek et al., 2001; Karakas and Gunduz, 2006; Otway et al., 2009). In addition, in cultured adipocytes, rhythmic treatment with melatonin has been shown to synchronize their metabolic and hormonal function, including leptin secretion (Alonso-Vale et al., 2008). This result perfectly fits with our *in vivo* demonstration that in hamsters without circulating melatonin at night, the daily rhythm of leptin is blunted. These findings highlight that rhythmic melatonin is a time-giver for rhythmic secretion of leptin. To firmly demonstrate that interpretation, further experiments in pinealectomized hamsters treated with melatonin are needed. It is expected that in contrast to continuous melatonin replacement, only restoration

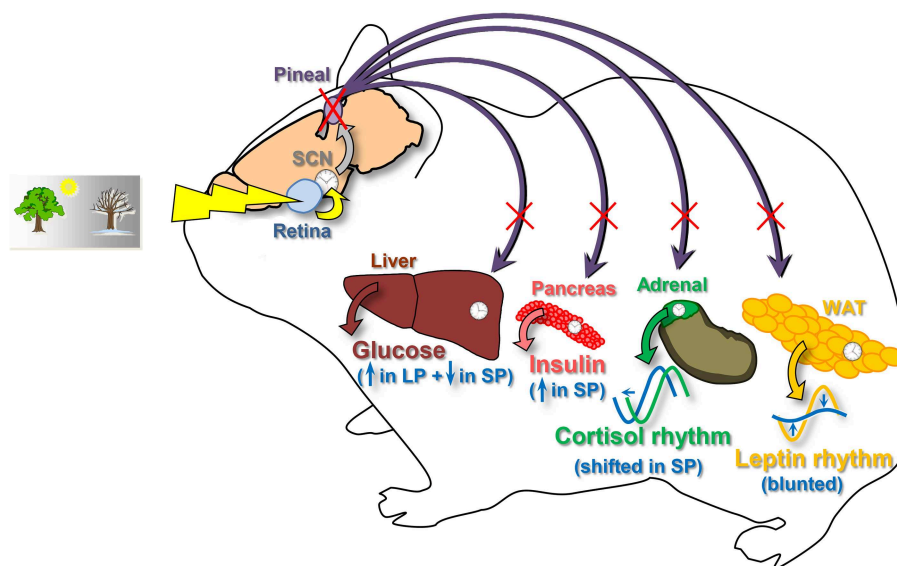


FIGURE 5 | Schematic view summarizing the effects of pinealectomy on metabolic physiology in the Syrian hamster. For each parameter, changes after pinealectomy are drawn in blue. Pinealectomy in Syrian hamsters suppresses the daily rhythmicity of plasma leptin synthesized by the white adipose tissue (WAT), whatever the photoperiod. Pinealectomy leads to shifts of the daily rhythm of

plasma cortisol in hamsters adapted to a short photoperiod. Furthermore, pinealectomy leads to increased levels of insulin in hamsters exposed to a short photoperiod (SP). Finally, pinealectomy increases plasma glucose in leaner hamsters exposed to a long photoperiod (LP), and decreases glycemia in fatter animals adapted to a short photoperiod (SP). SCN, suprachiasmatic nuclei, site of the master clock.

of a melatonin rhythm will reinstate daily variations of plasma leptin.

Organ-Specific Sensitivity to Rhythmic Melatonin or Glucocorticoids

Because of the presence of melatonin receptors in a multitude of central and peripheral organs, the daily rhythm of plasma melatonin is thought to distribute temporal cues generated by the master clock throughout the body, thus playing a role of coupling between the central and secondary clocks (Pevet and Challet, 2011). In the present study, we noticed that pinealectomy affects daily rhythms of plasma leptin, cortisol, and insulin in different ways depending on the rhythm considered (Figure 5). We suggest that these differential effects of melatonin (or its absence) *in vivo* are indicative of direct effects on targeted peripheral tissues, rather than indirect effects on upstream structures (e.g., in the central nervous system). Previous studies on organs (white adipose tissue, adrenal gland or pancreas) isolated *in vitro* are in accordance with this hypothesis (Peschke and Peschke, 1998; Alonso-Vale et al., 2008; Valenzuela et al., 2008).

It is worth reminding that rhythmic glucocorticoids, such as cortisol and corticosterone, are also considered themselves as internal time-givers. The prevalent view is that most, if not all, peripheral organs that express glucocorticoid receptors are sensitive to the synchronizing effects of applications of glucocorticoid agonist (dexamethasone) (Balsalobre et al., 2000). Therefore, a given structure expressing the *ad hoc* receptors could theoretically be sensitive to the two hormonal synchronizers. Accordingly, in the white adipose tissue, both glucocorticoids and melatonin can modulate timing of daily profiles of metabolic

gene expression (Alonso-Vale et al., 2008; Su et al., 2015), or hormonal output (i.e., leptin; Alonso-Vale et al., 2008; this study). In fact, this dual sensitivity may not be so widespread.

Some tissues, indeed, appear to be more specifically sensitive to the synchronizing effects of either melatonin or glucocorticoids. In the mouse liver for instance, glucocorticoids play a strong synchronizing effect, as shown by arrhythmicity in metabolic gene expression after adrenalectomy (Oishi et al., 2005), while melatonin cues do not markedly affect hepatic timing (Houdek et al., 2015). In the brain of mice, the daily variations of dopamine in the striatum are controlled by rhythmic melatonin, as evidenced by disappearance of rhythmicity after pineal ablation and restoration with daily injections of melatonin, while adrenalectomy does not impair the timing of the dopaminergic variations (Khaldy et al., 2002). Conversely, daily variations of serotonin in the rodent brain, known to be controlled by rhythmic glucocorticoids (Segall et al., 2006; Malek et al., 2007), are not affected by pinealectomy or daily injections of melatonin (Khaldy et al., 2002). Finally, circadian timing in the pars tuberalis of the hypophysis appears to be driven by circulating melatonin (Dardente, 2007).

These data, together with our differential findings on plasma cortisol and leptin after pinealectomy, favor the hypothesis that rhythmic melatonin acts as an internal synchronizer on targeted structures. The functional implication of these specific temporal regulations for physiology and health remains to be investigated. The seasonal species that display annual and reversible changes in metabolic physiology, like Syrian hamsters studied here, provide promising models in that respect.

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Local modulation of steroid action: rapid control of enzymatic activity

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Estrogens can induce rapid, short-lived physiological and behavioral responses, in addition to their slow, but long-term, effects at the transcriptional level. To be functionally relevant, these effects should be associated with rapid modulations of estrogens concentrations. 17 β -estradiol is synthesized by the enzyme aromatase, using testosterone as a substrate, but can also be degraded into catechol-estrogens via hydroxylation by the same enzyme, leading to an increase or decrease in estrogens concentration, respectively. The first evidence that aromatase activity (AA) can be rapidly modulated came from experiments performed in Japanese quail hypothalamus homogenates. This rapid modulation is triggered by calcium-dependent phosphorylations and was confirmed in other tissues and species. The mechanisms controlling the phosphorylation status, the targeted amino acid residues and the reversibility seem to vary depending of the tissues and is discussed in this review. We currently do not know whether the phosphorylation of the same amino acid affects both aromatase and/or hydroxylase activities or whether these residues are different. These processes provide a new general mechanism by which local estrogen concentration can be rapidly altered in the brain and other tissues.

Keywords: aromatase, hypothalamus, 17 β -estradiol, catechol-estrogens, phosphorylation, neurosteroidogenesis

Introduction

The local conversion of androgens into estrogens in specific areas of the central nervous system is an essential step in the activation of numerous testosterone-dependent physiological and behavioral processes, including sexual differentiation of the brain, negative feedback on the secretion of gonadotrophin releasing hormone and the activation of male sexual and aggressive behaviors (Beyer et al., 1976; MacLusky and Naftolin, 1981; Bagatell et al., 1994; Fisher et al., 1998; Honda et al., 1998; Toda et al., 2001; Rochira et al., 2006; Trainor et al., 2006). This transformation is catalyzed by the enzyme aromatase, also known as CYP 19 or estrogen synthase. In most vertebrates, central aromatase expression is restricted to specific neuronal populations mainly located in the hypothalamic/preoptic areas including the medial preoptic nucleus and in the limbic system (Roselli et al., 1985; Foidart et al., 1995a,b; Wagner and Morrell, 1997; Trainor et al., 2007; Metzdorf et al., 1999; Roselli and Resko, 2001). It should be noted that songbirds have an additional population of aromatase expressing neurons in the caudomedial nidopallium, in the telencephalon (NCM; Shen et al., 1995; Saldanha et al., 2000). Aromatase is also abundantly expressed in the whole brain of teleost fishes but only in glial cells

(Forlano et al., 2001, 2006; Diotel et al., 2010). The presence of aromatase in defined brain areas allows for local increase in estrogens (17 β -estradiol or E2) without affecting the periphery or surrounding brain regions. The estrogens resulting from local aromatization bind to estrogen receptors α or β , trigger a conformational change and dimerization of the receptor, and lead the receptor to translocate to the nucleus. The receptor dimer then binds to a specific response element on the DNA, usually but not only, upstream of the target gene, recruits a set of transcriptional coregulators and enhances or reduces gene transcription. This modulation of transcription affects the physiology of the cell and ultimately the functioning of the organ and the entire organism. The actions of estrogens at this genomic level develop relatively slowly (several minutes but usually several hours to several days) and have long lasting effects on the physiology, from several days to a life time. However, numerous laboratories have now described estrogen-dependent physiological changes that occur much more rapidly and independently of mRNA transcription and protein synthesis. For example, an acute estrogenic treatment *in vitro* triggers the activation of numerous intracellular signaling pathways including phosphorylation of the mitogen-activated protein kinase (MAPK) and cAMP response element binding protein (CREB) and changes in intracellular calcium concentrations (Mermelstein et al., 1996; Moss et al., 1997; Kenealy et al., 2011; Roepke et al., 2005). In the brain, these modifications lead to modulations of neuronal activity (Joels, 1997; Moss et al., 1997; Rønnekleiv and Kelly, 2002; Abraham et al., 2003, 2004; Boulware et al., 2005) and can in some instance rapidly affect behavior in numerous species (Hayden-Hixson and Ferris, 1991; Remage-Healey and Bass, 2004; Dewing et al., 2007; Micevych and Mermelstein, 2008; Lord et al., 2009; Seredynski et al., 2013; see Cornil et al., 2012a for review). These observations led to the hypothesis that estrogens are not only slow acting signaling molecules, or hormones, but can also function more rapidly like classical neurotransmitters, such as glutamate or dopamine (Balthazart and Ball, 2006; Saldanha et al., 2011).

While these rapid, non-genomic, effects of estrogens are currently the focus of intense research, they raise the question of how rapid increases of estrogens can occur. Indeed, estrogens, like other steroids, cannot be stored in synaptic vesicles before rapid release due to their lipophilic nature. We therefore suggested that the fast effects of estrogens require a rapid change of local steroid concentration via rapid changes in their synthesis rate by androgen conversion (Balthazart and Ball, 2006; Cornil et al., 2006), therefore implicating changes in aromatase activity (AA). Changes in AA often reflect changes in aromatase protein concentration resulting from relatively slow transcriptional control. For example, aromatase expression in the hypothalamus in most vertebrates is controlled by sex steroids: weak aromatase expression is detected in castrated males, while testosterone replacement markedly increases aromatase transcript, protein and activity (Roselli and Resko, 1984, 1989; Schumacher and Balthazart, 1986; Fusani et al., 2001). These changes in AA generally occur after several hours or days of treatment. However, the rapid effects of E2 introduced above require mechanisms affecting the synthesis of the steroid more rapidly, suggesting posttranslational change(s) of the protein activity.

Rapid Modulation of Aromatase Activity

The first evidence that AA is rapidly modulated was obtained in preoptic area/hypothalamus explants from Japanese quail. A rise in extracellular K⁺ concentration or an increase of intracellular calcium concentration rapidly (5 min) and transiently reduced AA (Balthazart et al., 2001b). These results suggested that the K⁺-induced transient depolarization resulted in an increase of Ca⁺⁺ from intracellular stores and led to a rapid reduction of AA. In agreement with this hypothesis, the inhibition of enzymatic activity was significantly hindered when the K⁺-induced depolarization was performed in a Ca⁺⁺-free medium (Balthazart et al., 2001b). In a different model, the zebra finch telencephalon, Remage-Healey and colleagues showed that a K⁺-induced depolarization and the increase of intracellular calcium concentrations were also able to significantly reduce AA, as suggested by the rapid reduction of E2 concentrations measured by ELISA on NCM microdialysates (Remage-Healey et al., 2011). However, the increase in calcium concentrations in this model resulted from the activation of voltage-gated calcium channels, and not from a release from intracellular stores. Indeed, the use of ω -conotoxin, a specific inhibitor of N-type Ca⁺⁺ channel, blocked the K⁺-induced decrease in E2 concentration (Remage-Healey et al., 2011).

Glutamate

Our group investigated physiologically relevant signaling molecules that could affect the local concentrations of calcium in the brain and investigated whether the activation of glutamate receptors could lead to a rapid modulation of AA in male quail preoptic/hypothalamic explants. We found that the glutamate agonists AMPA [2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid], kainate and, to a some extent, NMDA (N-methyl-D-aspartate) rapidly and reversibly down-regulated AA from quail hypothalamic explants. Addition of the glutamate receptor antagonist NBQX blocked the effect of kainate on AA, confirming the specificity of the treatments (Balthazart et al., 2006). Interestingly, intracellular recording combined with biocytin labeling of aromatase-positive neurons confirm that these neurons are sensitive to glutamate, a finding consistent with the idea that glutamatergic inputs on aromatase cells acutely regulate AA (Cornil et al., 2000, 2004). In parallel, *in vivo* retrodialysis of glutamate in the zebra finch telencephalon induced a significant reduction of local E2 concentration, while NMDA had no apparent effect (Remage-Healey et al., 2008).

Dopamine

It should be noted that dopaminergic signaling could also be involved in the rapid modulation of AA. Double immunocytochemistry highlighted a close interaction between dopaminergic fibers (tyrosine hydroxylase-positive structures) and aromatase-positive cells in the hypothalamus of Japanese quail (Balthazart et al., 1998). More importantly, several dopaminergic drugs rapidly inhibited AA in homogenates and *in vitro* explants obtained from the preoptic area hypothalamus of male Japanese quail (Baillien and Balthazart, 1997; Balthazart et al., 2002). This

inhibition was fully reversible in explant when the compounds were removed from the incubation medium (Absil et al., 2001). Surprisingly, agonists as well as antagonists of dopamine, specific for the D1 or D2 receptors, depressed AA in explants incubated *in vitro*, raising questions about the exact mechanism underlying the potential implication of D1 or D2 receptors. In addition, the observation that the dopamine reuptake inhibitor nomifensine had no effect in homogenates but strongly inhibits AA in explants suggests that the accumulation of dopamine within the synaptic cleft is the cause of AA inhibition.

GABA

Another neurotransmitter, the inhibitory amino acid GABA, did not affect the enzymatic activity in quail preoptic hypothalamus explants nor *in vivo* in zebra finch telencephalon (Balthazart et al., 2001b; Remage-Healey et al., 2008). It is therefore obvious that the direct links between the activation of the membrane receptor, calcium release and the exact intracellular pathway(s) involved in the rapid modulation of AA require further investigation.

Post-translational Modifications of Aromatase Activity by Phosphorylation

Post-translational modifications are a common mechanism involved in the control of protein activity and is a widely used regulatory mechanism in the brain to control for example NMDA receptor activity in hippocampal neurons (Chen and Roche, 2007) and tyrosine hydroxylase activity, the rate-limiting enzyme in catecholamine synthesis (Albert et al., 1984; Daubner et al., 1992). These phosphorylations are catalyzed by specific kinases that critically depend on the intracellular concentration of Ca^{++} and Mg^{++} . Interestingly, previous studies have implicated divalent cations in the control of AA (Ca^{++} : Onagbesan and Peddie, 1989; Mg^{++} : Steimer and Hutchison, 1991). We therefore wondered whether Ca^{++} and Mg^{++} , in addition of adenosine triphosphate ATP as a phosphate donor, during phosphorylation process could also potentially affect AA. We showed that exposure to ATP (2–8 mM), Ca^{++} (0.5–2 mM), and Mg^{++} (2–10 mM) rapidly and significantly inhibited AA in male Japanese quail preoptic area/hypothalamus homogenate (Balthazart et al., 2001b), strongly suggesting that phosphorylations rapidly inhibit this enzymatic activity. To test whether ATP/Mg/Ca conditions induce kinase-dependent protein phosphorylations or whether these conditions result in a non-specific inactivation of AA, we tested effects of the second messengers cAMP and cGMP, respectively activators of protein kinase A, PKA and protein kinase G, PKG. These kinase activators did not modify AA in the absence or presence of ATP/Mg/Ca (Balthazart et al., 2003). We also tested the effects of numerous kinase inhibitors on the AA of preoptic area/hypothalamic homogenates in the presence or absence of ATP/Mg/Ca (Balthazart et al., 2003). Several of these inhibitors significantly blocked the inhibition of AA induced by the addition of ATP/Mg/Ca while others had no effect. The general serine/threonine (Ser/Thr) kinase inhibitor Staurosporine (STAU), and the general tyrosine (Tyr) kinase inhibitor genistein (GEN) significantly blocked the inhibition produced by ATP/Mg/Ca

while they had no effect on basal AA. Similar effects (blockade of inhibition) were also observed in presence of the PKC inhibitor bisindolylmaleimide, the myosin light chain kinase inhibitor ML7, the inhibitor of PKA and PKC H7, the specific inhibitor of PKA/PKG H9, the PKA inhibitor H89 and the calmodulin antagonist trifluoperazin. Altogether, these data indicate that rapid modulation of AA results from the phosphorylation of both Tyr and Ser/Thr residues (Balthazart et al., 2001a, 2003). It should be noted that the potential quantitative role of each kinase family in the rapid modulation of AA in homogenates seems to vary according to the sex: While the addition of protein kinase inhibitors blocked the ATP/Mg/Ca-dependent inhibition AA in both sexes, the magnitude of these effects were sexually differentiated and usually more pronounced in females compared to males (Konkle and Balthazart, 2011). Moreover, phosphorylating conditions affected AA differentially in 2 subcellular compartments isolated via differential ultracentrifugation from zebra finch telencephalon. The reduction of AA by phosphorylating conditions was significantly more pronounced in the fraction containing synaptosomes (P2), as compared to microsomes (endoplasmic reticulum, P3 fractions, Cornil et al., 2012b).

We further investigated whether the rapid inhibition of AA by ATP/Mg/Ca-dependent phosphorylation processes is specific to the neuronal environment or can be observed in other aromatase-rich tissues. Effects of phosphorylating conditions were quantified in ovary and ovarian follicles homogenates from Japanese quail and demonstrated a drastic decrease in enzymatic activity within 15 min (Charlier et al., 2011). We also transiently and stably expressed human aromatase in HEK293 cells. Similarly to what was observed in the preoptic area/hypothalamus of Japanese quail and telencephalon of zebra finch, a K^{+} -induced depolarization triggered a pronounced but transient inhibition of AA expressed by transfected HEK293 cells. In addition, we also showed that phosphorylating conditions did not affect the enzyme affinity for the substrate but only changed the maximum velocity of reaction. Interestingly, the rapid enzymatic inhibition induced by depolarization also involved the activity of protein kinases as the addition of staurosporine (Ser/Thr kinase inhibitor) or Genistein (Tyr kinase inhibitor) blocked the effect of K^{+} -induced depolarizations on AA (Charlier et al., 2011). These observations are contrasting to what is observed in the breast cancer cell line MCF-7. Indeed, Catalano et al. (2009) showed that E2 rapidly modulates c-Src kinase action on aromatase tyrosine residues to enhance AA. It should however be noted that these effects were observed after 5 h of incubation and might be underlying different physiological processes.

In both cases however, the rapid change in AA is reversible, suggesting (1) that phosphorylation does not involve protein degradation when AA is reduced (Charlier et al., 2011) and (2) phosphatase is required to restore the un-phosphorylated stage. Interestingly, the general inhibitor of protein phosphatases (PPase), vanadate, did not significantly increase AA but reduced it in male quail preoptic area/hypothalamic homogenates. In addition, alkaline PPase inhibited AA in a dose-dependent manner in the presence, as well as in the absence, of ATP/Mg/Ca (Balthazart et al., 2005). In contrast, preincubation with acid

PPase completely blocked the inhibitory effects of ATP/Mg/Ca on AA although the addition of the PPase was unable to restore AA after the enzymatic activity had been inhibited by the phosphorylating conditions. Moreover, high concentrations of acid PPase also moderately inhibited AA in the absence of ATP/Mg/Ca (Balthazart et al., 2005). It should be noted that recent work on human placental choriocarcinoma cell line JEG-3 demonstrated that microsomal aromatase was rapidly inactivated with calcium, magnesium, and ATP, similarly to what is observed in quail preoptic area/hypothalamus homogenate and transfected cell lines, but the protein was then subsequently degraded in these conditions (Hayashi and Harada, 2014). More specifically, aromatase was protected from protein degradation by treatment with alkaline phosphatase, whereas degradation was enhanced by treatment with the phosphatase (calcineurin) inhibitors FK506 and cyclosporin A. Furthermore, aromatase was protected from degradation by treatment with kinase inhibitors, especially the calcium/calmodulin kinase inhibitors KN62 and KN93 (Hayashi and Harada, 2014).

It is therefore possible aromatase can first be rapidly inactivated by phosphorylation, then follow 2 different paths: either (1) its activity is restituted by dephosphorylation following interaction with phosphatase, or (2) the absence of dephosphorylation leads to subsequent degradation, i.e., a permanent inactivity of the enzyme. However, the specific enzymes involved in the phosphorylation-dephosphorylation mechanisms associated to the rapid and reversible control of AA are not fully defined and are currently under further investigation. It should also be noted that tyrosine phosphatase PTP1B was shown to reduce AA in MCF-7 and ZR75 breast cancer cells (Barone et al., 2012). Altogether, this suggests that some level of phosphorylation is required for a fully functional activity but future work should define in more detail the intricate role of phosphatases and kinases in the control of AA in different cell types.

Evidence for the Direct Phosphorylation of the Aromatase Protein

Our next question was to define whether phosphorylations controlling enzymatic activity directly affect the aromatase itself or modulate the activity of a cofactor that could secondarily regulate AA. We purified by immunoprecipitation the aromatase protein from quail preoptic area/hypothalamic homogenates and the presence of phosphorylated residues on the purified protein was investigated by Western blotting using anti-phosphoserine, anti-phosphothreonine or anti-phosphotyrosine antibodies. Confirming the pharmacological finding described above, phosphorylated Ser, Thr, or Tyr were identified by the specific antibodies at the level of the electrophoresis band corresponding to aromatase and the intensity of the phosphorylation signal was denser when homogenates were pre-incubated with ATP/Mg/Ca as compared to homogenates incubated in control conditions (Balthazart et al., 2003).

Similar experiments were performed on engineered human aromatase containing a c-myc tag that allows its specific immunoprecipitation. HEK293 cells transfected with this

construct were incubated with [γ - 32 P]-ATP in phosphorylating or non-phosphorylating (control) conditions. The radioactive aromatase c-myc in phosphorylating conditions was clearly observed at the expected molecular weight while only a low-intensity band was present in control conditions (Charlier et al., 2011). In additional experiments, the immunoprecipitated aromatase obtained after a 5 min-incubation of the cell lysate in phosphorylating conditions was clearly visible at the expected size on immunoblot when visualized with anti-phosphoserine antibody, while that band was not present in different control conditions (Charlier et al., 2011). This set of experiments clearly supports the hypothesis that the aromatase protein itself is rapidly phosphorylated in the presence of ATP/Mg/Ca, leading to a reduction of its enzymatic activity. Similarly, immunoprecipitation of His6-tagged aromatase in MCF-7 cells and investigation of phosphorylated amino acid by immunoblotting revealed that aromatase tyrosine residue is directly targeted by the kinase c-Src (Catalano et al., 2009, 2014).

Identification of Aromatase Residues Involved in the Rapid Control of Activity

This set of pharmacological experiments on quail hypothalamus explants and homogenates and the manipulations performed with immunoprecipitated human aromatase indicated that the inhibition of AA by phosphorylation is likely to be catalyzed by the activity of two Ser/Thr kinases, protein kinase A (PKA) and C (PKC), although the implication of tyrosine kinase and other Ser/Thr kinases cannot be completely ruled out (Balthazart et al., 2003; Charlier et al., 2011). The quail and human aromatase protein sequence were analyzed using bioinformatic tools to identify the potential phosphorylation sites, highly conserved amongst several avian and mammalian species. Fifteen and nineteen putative phosphorylation sites were identified for quail and human sequences respectively using the open access NETPHOS 2.0 program. These sites included 10 of the 27 serines, 5 of the 24 threonines, and 4 of the 17 tyrosines residues on the human sequence and we paralleled these findings with the results obtained using NETPHOSK, highlighting consensus sequences corresponding to PKC and PKA, protein kinases shown to affect quail and human AA during the pharmacological experiments (Balthazart et al., 2003; Charlier et al., 2011). The output pointed to 6 different residues, five serines (S118, S167, S247, S267, S497), and one threonine (T143) on the human sequence. It should be pointed out that other kinases might also be involved, as consensus binding sequence were observed on both quail and human aromatase. These were however not investigated. From this analysis, we decided to focus our attention on 6 different residues: S247, S267, and S497 that had high scores in both the predictive phosphorylation sites and PKA or PKC recognition consensus sequence; T462 and T493 that correspond to positions S455 and S486 in quail aromatase, two 2 residues that were predicted to be involved in the phosphorylation of aromatase (Balthazart et al., 2003). In addition, *in vitro* studies of the mouse aromatase performed by 2 different groups suggested that 2 other amino acid residues, namely serine S118 (Miller et al., 2008) or tyrosine

Y361 (Catalano et al., 2009) can be phosphorylated. Indeed, the phosphorylation of Y361 triggered by the tyrosine-kinase c-Src seems involved in the E2-dependent upregulation of AA in breast cancer cell lines (Catalano et al., 2009) while phosphorylation of S188 by AGC-like kinases, including PKC and PKA, seems important for the stabilization of the protein, and therefore for the expression of AA (Miller et al., 2008). It should be noted that the effects of the phosphorylation status of these 2 residues on the rapid change of AA was not investigated and we explored the potential implication of S188 phosphorylation (not Y361) in the rapid control of AA.

Altogether, we investigated the consequence of mutating 6 S/T residues into alanine (A) using the human aromatase as template. All mutants expressed normal baseline AA and pre-incubation with ATP/Ca/Mg for 15 min markedly reduced this enzymatic activity in the 6 different mutants alone (S118A, S247A, S267A, T462A, T493A, S497A) or in combination (S267A-S247A, T493A-T462A-S267A, T493A-T462A-S267A-S247A, S497A-T493A-T462A-S267A-S247A) roughly to the same extent as in wild type enzyme, clearly showing that these single or combined mutations did not block the rapid inhibition of aromatase by phosphorylating conditions. The absence of effect, against all expectations, was thoroughly discussed in the original article (Charlier et al., 2011). In summary, it is likely that a combination of several phosphorylated residues that was not tested here is required to control AA. The control by multiple kinases reinforces the idea that several amino acids must concomitantly be phosphorylated to modify AA. It is possible

that other consensus sites for phosphorylations and for other types of kinases that were also predicted on the quail and human aromatase sequences might be involved. Alternatively, it is possible that the control of AA by phosphorylations depends on the modulation of certain factors interacting with aromatase, in addition to the enzyme itself. Aromatase requires the presence of an active NADPH-cytochrome P450 reductase for electron transfer (Thompson and Siiteri, 1974; Miller, 2005; Hong et al., 2009). The phosphorylation of the reductase could be a control mechanism affecting the electron transfer and this idea should be further investigated.

Behavior and Rapid Modulation of Aromatase Activity

It is important to note that the change in enzymatic activity resulting from the phosphorylation of aromatase is physiologically and behaviorally relevant. Indeed, rapid changes in AA were demonstrated to occur *in vivo* in response to social interactions in a few species investigated to date, including birds and fish. For example, expression of appetitive or consummatory sexual behavior rapidly reduces AA in specific brain regions in the preoptic-hypothalamic area of quail after 5–30 min of sexual interaction with a receptive female (Cornil et al., 2005a; de Bournonville et al., 2013) while exposure to acute restraint stress, or stress-related hormones such as AVT and corticosterone lead to an increase in AA in both male and female quail (Dickens et al., 2011, 2013). Male removal from a socially stable group of the

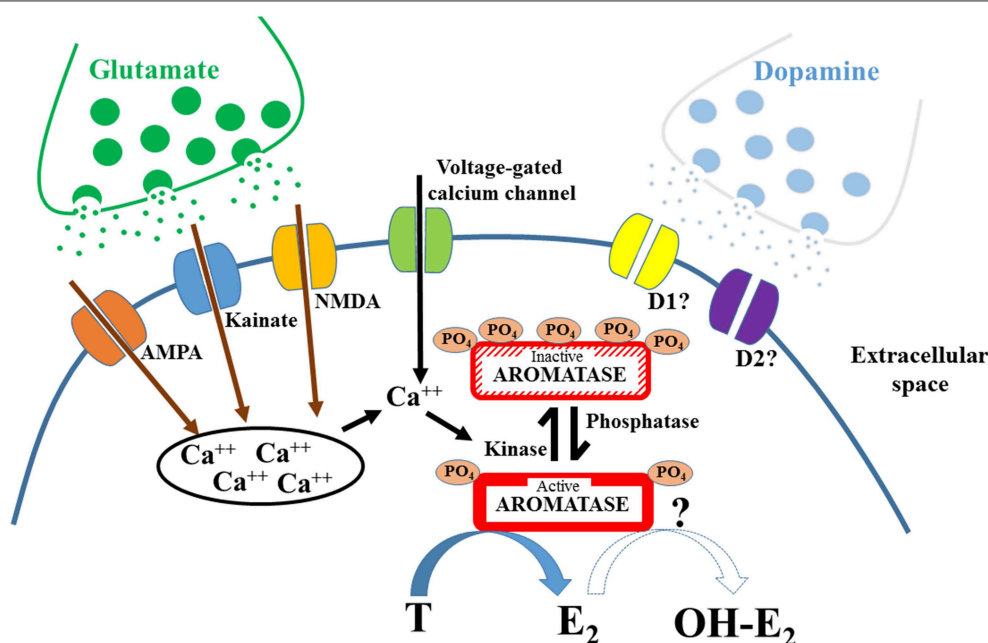


FIGURE 1 | Schematic diagram representing the mechanisms involved in the rapid control of aromatase activity.

Phosphorylations (PO_4) rapidly modulate aromatase activity, inhibiting the transformation of testosterone (T) into 17β-estradiol (E_2). It is likely that these modifications are induced by calcium-voltage channels, by

glutamatergic receptors and/or by dopaminergic receptors. The increase of intracellular calcium (Ca^{++}), either from intracellular storage or from the activation of voltage-gated channel is in most cases a prerequisite for the inhibition of aromatase activity. Change in phosphorylation level could also affect the hydroxylase activity of aromatase.

sex-changing fish *Lythrypnus dalli* resulted in rapid increases in aggression associated with a rapid reduction in brain AA in the dominant female (Black et al., 2005, 2011). Conversely in zebra finches, a brief exposure to song (30 min) resulted in an increase of AA in NCM within the telencephalon (Remage-Healey et al., 2009). While behavior can affect AA, the reverse is also true and recent studies showed that rapid changes in AA modulate behavior and sensory processing. Indeed, an acute systemic or central administration of aromatase inhibitors such as Vorozole or Androstatrienedione significantly and rapidly reduced male sexual behavior in quail and mice (Cornil et al., 2005b; Taziaux et al., 2007; Seredynski et al., 2013), approach behavior toward females in male goldfish (Lord et al., 2009), aggressive behavior in Beach and California mice (Trainor et al., 2007, 2008) and vocalizations in midshipman fish (Remage-Healey and Bass, 2004). Fadrozole, another aromatase inhibitor, rapidly suppressed the preference for a bird's own song in male zebra finches (Remage-Healey et al., 2010) and reduced song-elicited neuronal firing rate in zebra finches and prevented the expression of hearing-driven gene expression in NCM, a brain regions involved in song discrimination in songbirds (Tremere et al., 2009). Altogether, this clearly confirms that the rapid modulation of AA, likely by phosphorylation, is of physiological importance. This also shows that the rapid inhibition of AA rapidly influences the physiology and behavior.

Catabolism of Estrogens

Therefore, there must exist some mechanisms that are able to rapidly clear locally-produced estrogens to terminate their effects. The half-life of E2 in the brain is not known, although calculations derived from pharmacokinetic data estimated that its half-life in the blood is below 30 min (Johnson and van Tienhoven, 1981; Tsang and Grunder, 1984). The liver is known to express several enzymes involved in the clearance of circulating estrogens, notably CYP1A1, CYP1A2, CYP1B1, and CYP3A (Whirl-Carrillo et al., 2012). These enzymes catalyze the conversion of estrogen into inactive (or less active) water-soluble metabolites by oxidative metabolism and allow for further sulfation by sulfotransferases, glucuronidation by glucuronyltransferases, or methylation by catecholmethyltransferases. The elimination of plasma estrogens from the organism is ensured through excretion following their conjugation. Although this type of degradation of estrogens occurs mainly in the liver, some catabolic activity is also observed in the brain, albeit to a much lower extent. We hypothesized that the rapid elimination of estrogens in the brain involves the enzyme aromatase itself. Indeed, a few studies suggest that, in addition to its estrogen synthase activity, aromatase also catalyzes estrogen hydroxylation. A previous study in Japanese quail showed that 2-hydroxylase enzymatic activity is present in all brain regions that are known to contain a high level of AA, including the median preoptic nucleus and the ventromedial nucleus of the hypothalamus (Balthazart et al., 1994), suggesting that the same protein is involved in both the production and the conversion of estrogens. However, no sex difference in 2-hydroxylase activity was identified here, while AA is higher in males than in females in almost every brain region.

The first evidence that aromatase protein also had estrogen hydroxylase activity came from Osawa et al. (1993). They demonstrated that purified aromatase obtained from placental microsomes demonstrates an estrogen-2-hydroxylase activity in certain conditions. Both aromatase and estradiol 2-hydroxylase activities were observed after purification of human placental microsomal cytochrome P-450 by monoclonal antibody-based immunoaffinity chromatography and gradient elution. Moreover, they confirmed the concomitant existence of estradiol hydroxylase activity with AA by expressing human aromatase in CHO cells. Not only did the purified aromatase exhibit the hydroxylase activity but testosterone and androstenedione competitively inhibited estradiol 2-hydroxylation, and conversely, estrone and estradiol competitively inhibited aromatization of both testosterone and androstenedione. Similarly, equine aromatase expressed *in vitro* has a clear hydroxylase activity (Almadhidi et al., 1996). A specific antiserum raised against purified testicular equine P450arom and known to inhibit AA was also found to inhibit the estrogen hydroxylase activity of equine placental microsomes. Furthermore, the estrogen hydroxylase activity was inhibited in a dose-dependent manner by different aromatase inhibitors, including fadrozole and 4-hydroxyandrostenedione (Almadhidi et al., 1996). It remains to be determined whether the phosphorylation of the same residue(s) leads to the rapid reduction of both estrogen synthase and estrogen hydroxylase activity.

Conclusions

Altogether, a few laboratories have accumulated strong evidence showing that AA can be rapidly modulated via post-translational modifications, most notably via phosphorylation (see **Figure 1** for general model). The rapid modulation of AA by phosphorylating conditions is a widespread mechanism present in different tissues affecting aromatase from various species, including humans. The mechanisms leading to these modifications remain poorly understood, but it is increasingly clear that the enzymatic changes must result in a local rapid modulation of estrogens availability and consequently in a modification of cellular estrogen-dependent events that are not mediated by the genomic actions of these steroids. The phosphorylation/dephosphorylation processes provide a new widespread mechanism by which estrogens concentration could be rapidly altered in the brain and other tissues, albeit likely in different directions. It would be interesting to test whether precise phosphorylation patterns affect one type of enzymatic activity while leaving the other untouched or whether phosphorylation is a general shut-down switch for all enzymatic activity displayed by the aromatase protein.

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Farnesol-like endogenous sesquiterpenoids in vertebrates: the probable but overlooked functional “inbrome” anti-aging counterpart of juvenile hormone of insects?

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Literature on the question whether the juvenile stage of vertebrates is hormonally regulated is scarce. It seems to be intuitively assumed that this stage of development is automated, and does not require any specific hormone(s). Such reasoning mimics the state of affairs in insects until it was shown that surgical removal of a tiny pair of glands in the head, the *corpora allata*, ended larval life and initiated metamorphosis. Decades later, the responsible hormone was found and named “juvenile hormone” (JH) because when present, it makes a larva molt into another larval stage. JH is a simple ester of farnesol, a sesquiterpenoid present in all eukaryotes. Whereas vertebrates do not have an anatomical counterpart of the *corpora allata*, their tissues do contain farnesol-like sesquiterpenoids (FLS). Some display typical JH activity when tested in appropriate insect bioassays. Some FLS are intermediates in the biosynthetic pathway of cholesterol, a compound that insects and nematodes (=Ecdysozoa) cannot synthesize by themselves. They ingest it as a vitamin. Until a recent (2014) reexamination of the basic principle underlying insect metamorphosis, it had been completely overlooked that the Ca²⁺-pump (SERCA) blocker thapsigargin is a sesquiterpenoid that mimics the absence of JH in inducing apoptosis. In our opinion, being in the juvenile state is primarily controlled by endogenous FLS that participate in controlling the activity of Ca²⁺-ATPases in the sarco(endo)plasmic reticulum (SERCAs), not only in insects but in all eukaryotes. Understanding the control mechanisms of being in the juvenile state may boost research not only in developmental biology in general, but also in diseases that develop after the juvenile stage, e.g., Alzheimer’s disease. It may also help to better understand some of the causes of obesity, a syndrome that holometabolous last larval insects severely suffer from, and for which they found a very drastic but efficient solution, namely metamorphosis.

Keywords: farnesol, insect hormones, sex steroids, development, puberty, Alzheimer, *Caenorhabditis elegans*, obesity

INTRODUCTION

All animals, plants and fungi pass through a juvenile stage before becoming reproductively active and next becoming aged. In holometabolous insects a high titer of juvenile hormone (JH), an ester of the endogenous sesquiterpenoid alcohol farnesol (Figure 1) keeps larvae in their juvenile state. JH can occur in several isoforms¹ (1). It makes a larva molt into another larval stage. This is known as the “status quo effect” of JH. When the JH titer drops to zero at the end of larval life, metamorphosis is initiated. When metamorphosis nears completion, the JH titer rises again and the enclosing adult enters or completes (if the gonads started developing already during metamorphosis) the reproductive phase, and concurrently ages rapidly.

Hitherto, it was assumed that vertebrates do not have such a juvenilizing hormone, or, if they do have one, thyroid hormones, in particular triiodothyronine (T3) is likely candidates. Like the absence of JH has a key role in initiating metamorphosis in all holometabolous insects, the presence of T3 has a prominent role in initiating metamorphosis in some fish and amphibian species (3). Remarkably T3 has been shown to be active in some insect bioassays (4, 5). However, functional evidence supporting this view remains unconvincing (2). In particular the lack of a suitable bioassay(s) which is indispensable for monitoring the successive steps in the purification procedure represents the bottleneck for identifying the JH of vertebrates, if it exists. As long as this situation continues, one has no choice but exploring the possibilities of comparative endocrinology between vertebrates and invertebrates for finding an alternative strategy for unraveling the control mechanisms for initiating and maintaining the juvenile state in vertebrates.

¹ http://en.wikipedia.org/wiki/Juvenile_hormone

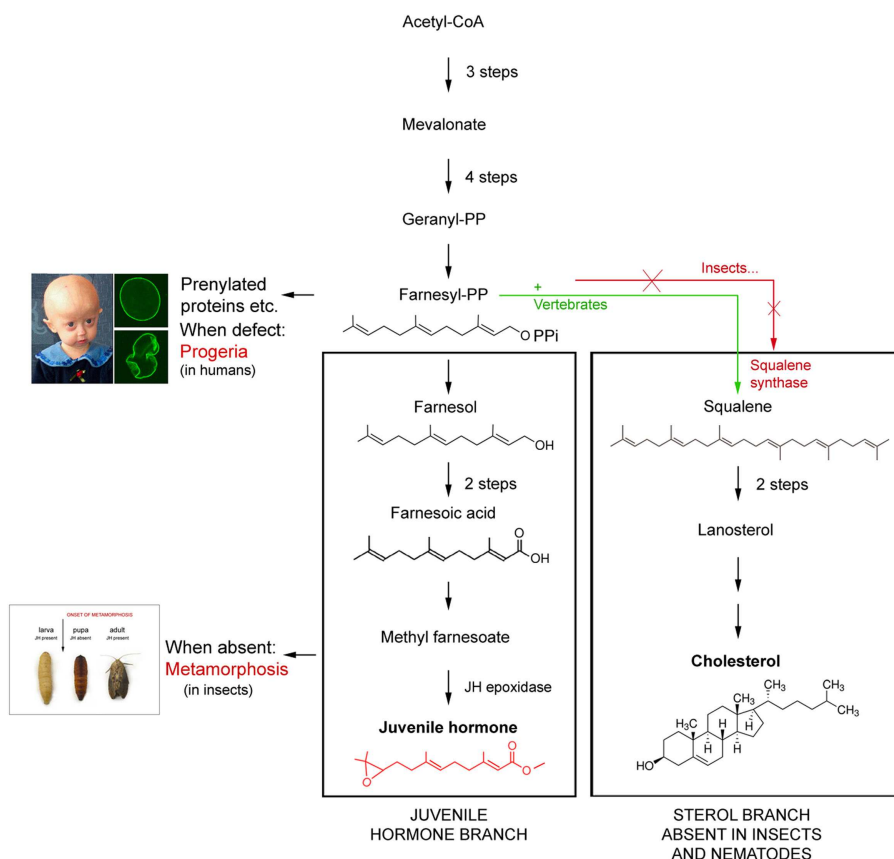


FIGURE 1 | Schematic representation of the difference in the mevalonate-based biosynthetic pathway in insects and nematodes (=Ecdysozoa) on one hand, and vertebrates on the other. The main difference is that Ecdysozoa cannot synthesize

cholesterol on their own because they miss the key enzyme squalene synthase. Whether their common ancestor lost the coding gene or never had it remains a matter of discussion. Modified after De Loof et al. (2).

The leading idea in this paper is that being in the juvenile state as an animal, follows from controlling principles that were already present in the common ancestor of plants, fungi, protostomian, and deuterostomian animals. Hence the signaling pathways involved are probably largely over a billion years old. It is therefore more likely that their key components have been well conserved in evolution, rather than that deuterostomes (to which vertebrates belong) “all of a sudden” invented a completely new strategy for generating and temporarily maintaining a juvenile state. It is well-known that insects, which are protostomes, use JH as their juvenilizing agent. Hence, the question is: could it be that in vertebrates, and by extension in all deuterostomes, the basic elements of this endogenous sesquiterpenoid system continue to be present and operate in a similar way, perhaps in a slightly modified form?

In this concept paper, we will argue that this is probably the case. We will also outline that research into aging-related diseases may benefit from the novel insights.

WORDING IN SHORT OF THE PARADIGM

Both deuterostomes and protostomes must have inherited the basic principles of passing through a juvenile state before becoming sexually mature from their common ancestor. These principles are probably still operational in both. Insects, in particular

holometabolous ones which in this respect are best studied, use JHs as their key hormone for realizing and maintaining the juvenile state. The Ca^{2+} -ATPase present in the sarco(endo)plasmic reticulum (SERCA) likely functions as the largely overlooked membrane receptor for JH and for at least some other endogenous farnesol-like sesquiterpenoids (FLS). No functional hormonal counterpart of JH has yet been identified in any vertebrate. Like all eukaryotes, vertebrates do have farnesol and some other FLS, but their role is only partially understood. We think that being a juvenile vertebrate depends upon the interaction of FLS with the Ca^{2+} -homeostasis system, like this is the case in insects. However, the key difference with insects is that in vertebrates farnesol/FLS is probably not a hormone. Instead, its precursor farnesyl-pyrophosphate (FPP) is present and probably synthesized in all cells of the body as one of the precursors in the cholesterol biosynthetic pathway. Because insects and nematodes either lost or never had the gene coding for the enzyme squalene synthase that converts squalene (that is formed from FPP) into cholesterol, they cannot make cholesterol by themselves. They solved this problem by ingesting cholesterol as a vitamin and by using a farnesol ester, namely JH as a circulating hormone to control at least one of the components of the Ca^{2+} -homeostasis system, namely the SERCA-pump system.

CONSTRUCTING THE PARADIGM

HOW IS THE JUVENILE STATE DEFINED?

A juvenile is an individual organism that has not yet reached its adult form, sexual maturity or size. In particular in holometabolous insects, the juvenile state corresponds to all larval stages before metamorphosis is initiated. In amniote vertebrates, the embryo represents the larval stage, and “juvenile” applies to the time between hatching/birth and reaching maturity.

Animals in which the transition from larva/juvenile to sexually mature adult takes only days (most insects) are better experimental models for studying the principles of being a juvenile than the ones in which this transition takes months to even years.

Extracts of mammalian tissues do contain “JH bioactivity”

In the pioneering decades (1960–1980) following the identification of the chemical nature of the two non-peptidergic key insect hormones, JH, and the steroid molting hormone ecdysone, various tissues from insects but also from other species, including mammals, were analyzed for the presence of compounds with JH-like activity in specific insect bioassays. Some remarkable results were obtained. In insects the most unexpected one was that not the *corpora allata*, tiny glands in the head thought to be the only site of JH synthesis, were the richest source of JH, but the adult male accessory glands of the moth *Hyalophora cecropia*, an important experimental model at that time. The huge JH concentrations present in these glands which are the physiological counterpart of the prostate gland of mammals enabled the purification of JH. It turned out to be an ester of farnesol. At that time, FPP was already known as a precursor of cholesterol in vertebrates. The fact that insects cannot synthesize cholesterol by themselves made researchers logically conclude that farnesol/FLS in insects only served as the precursor for JH. A second remarkable result was that Williams et al. (6) showed that extracts from several mammalian tissues (thymus, human placenta, and others) displayed activity when tested in typical bioassays for insect JH.

Analysis of the chemical nature of the JH-bioactive substance in extracts of mammalian tissues showed that the active factor was farnesol, the same as the active factor present in excrements of the mealworm *Tenebrio molitor* and in yeast (7, 8). Thus vertebrates do have farnesol, and this farnesol, along with other compounds, some of them being farnesol-like (9) is active in insect JH-bioassays. Yet, although this finding was intriguing, it was not further analyzed in depth because it seemed normal that a direct precursor of JH, of which the chemical identity was elucidated by Röller and Dahm (10), could also have moderate bioactivity. The data got forgotten because it was deemed irrelevant for vertebrate physiology. Moreover, one should keep in mind that in those days, the idea that the endocrine systems of vertebrates and invertebrates might have some elements in common, was far from readily accepted. To date, farnesol is best known in the perfume industry where it is used as an adjuvant. It was named after the Farnese acacia tree (*Vachellia farnesiana*) of which the flowers are a rich source of farnesol. Its exact role in plants remains to be elucidated.

LITTLE PROGRESS IN THE PAST 45 YEARS

To date, we know that the basic principles of the endocrine systems of both vertebrates and insects are very similar, probably because they were already present in their common ancestor. We also

have a good idea which mutations resulted in differences in their steroids, endogenous sesquiterpenoids, and peptide hormones (11, 12). Current vertebrates no longer have the genes/enzymes needed for synthesizing ecdysteroids which are probably more “ancient” than the vertebrate-type steroids. On the other hand, insects either never had (2, 13) or lost the enzymes needed for biosynthesizing most “modern vertebrate-type steroids”. Yet, some such “vertebrate-type” steroids like pregnenolone, testosterone, eestradiol, etc. are present in some insect species, but their function, if any, has not yet been found (14, 15). The biosynthetic pathway of FLS is well known (16) (Figure 1). However, the exact mode of action of JH is still a matter of discussion (2, 17–19).

NEVER TOTAL ABSENCE OF FARNESOL IN THE LIFE CYCLE OF VERTEBRATES

In insects there is a natural condition in which JH is totally absent, namely during metamorphosis of holometabolous insects. When synthetic JH is applied before metamorphosis is initiated, larval life is usually extended. Depending upon the species, one or more extra larval instars can be formed, proving that JH is indeed a hormone that maintains the juvenile state.

In vertebrates, the situation “zero farnesol/FLS,” a necessary tool for defining the role of farnesol is never realized during lifetime. This is due to the fact that unlike in insects where – in larvae – farnesol and JH are only synthesized in the *corpora allata*, FLS are present in all cells of the vertebrate body (Table 1, see later). This is probably the case throughout all developmental stages.

In vertebrate physiology, the question whether, perhaps, farnesol/FLS may have a function on its own, additional to that of FPP in prenylation and in serving as a precursor of cholesterol has not (yet) been an issue.

TOTAL ABSENCE OF JH IN INSECTS MIMICS SOME OF THE EFFECTS OF THE SERCA-PUMP BLOCKER THAPSIGARGIN

What happens in insects when the JH/farnesol titer drops to zero? In holometabolous insects total absence of the sesquiterpenoid JH

Table 1 | Occurrence of farnesol, its precursor farnesyl pyrophosphate (FPP) and its derivatives, farnesal and farnesoic acid in a variety of tissues of a male mouse (*Mus musculus*).

| | FPP | Farnesol | Farnesal | FA |
|----------------|-----|----------|----------|----|
| Brain | + | – | + | + |
| Muscle | – | + | + | + |
| Thymus | – | + | + | + |
| Salivary gland | – | + | + | + |
| Gut | – | + | + | + |
| Liver | – | – | + | + |
| Testis | + | + | + | + |
| Prostate | + | + | + | + |
| Blood | + | + | + | + |

Metabolites were extracted from about 20 mg of tissue and 5 μ l of blood.

FPP, farnesyl pyrophosphate; FA, farnesoic acid.

Meaning of other symbols: compared to values present the *corpora allata* of insects, the best studied tissue in this respect: –: close to the detection limit; +: relatively high concentrations; +: moderate concentrations.

is a regular part of the life cycle. Indeed, the *corpora allata*, the only site of farnesol and JH synthesis in juvenile insects become completely inactive at the end of larval life. Furthermore, all JH that still circulates in the hemolymph is also fully degraded by specific esterases. This results in the zero JH/FLS situation. Exactly this absence of JH rather than the release of the prothoracicotropic hormone (13) initiates metamorphosis. The most dramatic aspect of metamorphosis is the programmed cell death/apoptosis of the tissues that actively secrete proteins such as the fat body, the alimentary canal, the salivary- and prothoracic glands, etc. (2).

What happens in vertebrates when an endogenous FLS can no longer exert its normal function becomes apparent from the effects of administration of the plant toxin thapsigargin, which is, like farnesol/JH also a sesquiterpenoid. Thapsigargin blocks the SERCAs of both vertebrates and insects. This indicates that not only the overall structure of SERCAs has been very well conserved in evolution, but that the binding site of thapsigargin on the SERCAs has also been conserved. De Loof (20) argued that this binding site of sesquiterpenoids on the endoplasmic reticulum functions as a third type/family of receptors that complements the much better documented plasma membrane receptors and nuclear receptors for (some types) of hormonal ligands.

In thapsigargin-sensitive cells both the absence of JH as well as the administration of thapsigargin make the SERCAs stop pumping Ca^{2+} into the lumen of the smooth- and rough endoplasmic reticulum (SER/RER). As a result the Ca^{2+} -concentration in the cytosol increases up to the level that Ca^{2+} -induced apoptosis (21) is induced.

WHY DID NEMATODES, IN PARTICULAR *CAENORHABDITIS ELEGANS*, RETAIN THE GENES FOR SYNTHESIZING FARNESOL DURING HUNDREDS OF MILLIONS YEARS?

Like in insects, the enzyme squalene synthase which is essential for the biosynthesis of cholesterol is absent in nematodes. Either these Ecdysozoa lost the gene or they never had it (20). This situation probably dates from before the divergence of deuterostomian and protostomian animals, or, perhaps from even before the divergence of plants, fungi, and animals, thus from at least one billion years ago. Yet, nematodes still express the genes coding for the enzymes that are instrumental to the biosynthesis of farnesol, its precursors and some metabolites, but not of cholesterol (22, 23). The fact that these genes have been conserved for so long indicates that farnesol/FLS must have a function different from that of merely serving a role in the biosynthesis of cholesterol; otherwise the genes would have become redundant. In worm research, this question triggered some interest, but for lack of performing quantification methods of FLS in small samples, a conclusive answer has not yet been given. What could that missing function be? The comparative approach with insects may provide the answer.

We performed an orienting experiment with the method of Rivera-Perez et al. (24) which will be outlined next. The results were that all FLS listed in Table 1 are present in juvenile and adult *C. elegans*. In due time, they will be published elsewhere.

A NOVEL, HPLC-BASED METHOD FOR QUANTIFYING FARNESOL AND ITS PRECURSORS HAS RECENTLY BEEN DESCRIBED

The ancient data from Williams et al. (6) and of Schmialek (7) on the presence of farnesol in a few tissues of mammals did not

give any indication on its origin, either from the ingested food or from an endogenous site of synthesis. All cells of the animal body need cholesterol. A commonly held view (25) is that the few major production sites of cholesterol, namely the liver and the intestine, secrete enough cholesterol into the bloodstream to comfort all peripheral tissues. In this scenario, one should not find the typical precursors and some metabolites of farnesol in these peripheral tissues. The other possibility is that all tissues express the genes for biosynthesizing farnesol, its precursors and some derivatives, not so much because they would not receive sufficient amounts of cholesterol from the blood and thus to compensate for such theoretical insufficiency, but because they all need farnesol/FLS for another reason than for using it for cholesterol synthesis.

Sesquiterpenoids are notoriously difficult to quantify accurately and robustly because of their lipophilic and labile nature and their tendency to bind non-specifically. Mass spectrometry approaches could be accurate, but are expensive and complex to interpret (1, 24).

Fortunately, a novel, very sensitive method developed by Rivera-Perez et al. (24) allows analyzing the whole biosynthetic pathway, from mevalonate up to farnesol and some derivatives (further to JH if needed) at once. The assay is based on the derivatization of analytes with fluorescent tags, with subsequent analysis by reverse phase HPLC coupled to a fluorescent detector (HPLC-FD).

PRESENCE OF FRS IN MAMMALIAN TISSUES

To verify if the old data of Williams et al. (6) are correct, we performed an orienting experiment with the Rivera-Perez et al. (24) method using extracts of tissues of a male mouse (*Mus musculus*). Although the method is able to generate both qualitative and quantitative data, we have limited ourselves to the qualitative ones. The reason is that the imposed deadline for submission of this paper did not allow generating a sufficient number of replicate experiments for an accurate quantification of all intermediates. Nevertheless, the results were convincing enough to state that all analyzed tissues of a male adult mouse seem to have different metabolites in the FLS pathway, and by extrapolation the different necessary enzymes for biosynthesizing several FLS, as shown in Table 1.

Contrary to what could be expected, FPP the direct precursor in the biosynthesis of squalene is only present in high concentrations in brain tissue and less in tissues known to be actively synthesizing cholesterol. FPP is also important for prenylation. The presence of FPP, farnesol, farnesal, and farnesoic acid in blood indicates that a role as a hormone may not *a priori* be ruled out, on condition that none originates from the food. In the literature there are no data suggesting a hormonal role. The high concentrations of farnesoic acid are intriguing. Farnesol and farnesal are quite toxic molecules in living cells. Rizzo and Craft (26) who studied the Sjögren–Larsson syndrome, demonstrated that conversion of farnesal to farnesol by an aldoketo reductase reduces the toxicity. The produced farnesol leaks out of the cells. The same effect was observed in insects in the JH pathway; the excess of farnesal in the *corpora allata* is converted back to farnesol that can leak out of the gland (27).

These preliminary results urge for an in depth qualitative and quantitative study of the farnesol/FLS biosynthetic pathway in all tissues of the body throughout development.

INBROME SIGNALING VERSUS A ROLE AS A HORMONE

The term “inbrome” stands for “intramembrane signaling substance.” It was introduced by De Loof et al. (2) to denote and categorize chemicals that act in the plane of membranes by binding to receptors that are located in membranes, in particular in intracellular membranes. A typical example is the plant toxin thapsigargin that binds to the Ca^{2+} -ATPases located in the sarco(endo)plasmic reticulum.

In the past neither farnesol nor any other FLS has been advanced as a hormone of vertebrates. The fact that all tissues seem to have the biosynthetic machinery for some FLS, suggests that if these compounds do have a role in controlling some cell-physiological process, e.g., Ca^{2+} -homeostasis they can do so from within the cell, without the need of having to be secreted into the blood stream first. The fact that single-celled organisms, like yeast, produce sesquiterpenoids, including farnesol, via the mevalonate pathway (28) supports this view. The “inbrome model” may explain some aspects of the mode of action of lipophilic hormones, in particular of sex steroids and JH. There is a major problem with the widely accepted Karlson model of steroid hormone action (29). It says that a steroid (ecdysone in Karlson’s experiments) diffuses right through the plasma membrane “unnoticed” (=without changing the permeability or electrical properties of the plasma membrane), next through the cytoplasm where it binds to a cytoplasmic receptor (=a later finding), and that finally this complex passes the nuclear pore complexes into the nucleus and binds there to the promoter regions of specific genes. Very few researchers are aware of the fact that this model was not at all readily accepted at the time it was launched. To the contrary, it was heavily disputed for good reasons. The most convincing counterargument was that the so called “specific” hormone-induced puffs could as well be induced in the absence of any hormone by changing the ionic concentration of some inorganic ions in medium in which the salivary glands were incubated (2, 30).

Karlson’s model is too simple. It is much more probable that once a lipophilic signaling molecule enters a membrane, it can cause electrical changes at the level of the plasma membrane, and even more important, it will rapidly diffuse throughout the whole continuum of all interconnected intracellular membranes. Because the plasma membrane forms a continuum with the endoplasmic reticulum and the outer part of the nuclear envelope, this means that lipophilic signaling molecules are omnipresent in membranes. For figures illustrating this chain of events, see De Loof (20) and De Loof et al. (2). The action of steroid hormone-receptor complexes inside the nucleus, which has been thoroughly studied in the past decades, is only one of the aspects of steroid hormone action, not at all the full story.

THE JUVENILE STATE ENDS AT PUBERTY: WHY AND HOW?

At puberty the juvenile state transits into the reproductive state. In vertebrates, increased GnRH secretion causes increased synthesis and release of gonadotropins. In endocrinology the general consensus is that the gonadotropins LH and FSH “stimulate”

the production of sex-related steroids (progesterone, estrogens, androgens, etc.). However, another interpretation is possible. De Loof et al. (13) stated that the appearance of peak concentrations of ecdysteroids in the hemolymph of metamorphosing and prediapausing insects coincides with the programed cell death of several tissues, in particular those that actively secrete proteins during larval life. Therefore, the appearance of such steroid hormone peaks indicates that, somewhere in the body, some tissue(s) is undergoing apoptosis, e.g., the fat body of insects or the follicle cell layer surrounding growing oocytes. According to this view the primordial role of the gonadotropins FSH and LH is not the “stimulate” the ovarian follicle cells to engage in producing steroid sex hormones, but to force them to undergo apoptosis. This has also been suggested before in our concept that reproduction is in fact a strategy of the cellular defense system (31). Later, De Loof et al. (13) evaluated whether a similar mechanism with a key role for apoptosis might be operational in the context of the onset of insect metamorphosis which marks the end of the juvenile state.

From “puberty” on, egg production starts. Eggs accumulate yolk proteins, lipids, glycogen, and last but not least huge amounts of Ca^{2+} . The liver of vertebrates and its counterpart in insects, the fat body, secrete lots of yolk protein precursors, vitellogenins, into the bloodstream under the influence of female sex steroids. Any protein secretion through the RER–Golgi system is accompanied by the secretion of Ca^{2+} (20). A typical example is the production of milk in mammals. The Ca^{2+} -concentration in milk amounts to about 50 mM. This should be compared to the Ca^{2+} concentration in the cytosol of unstimulated cells in general, which is as low as 100 nM. The onset of puberty therefore involves a drastic change in Ca^{2+} -homeostasis, in particular in females. These changes have to be controlled one way or another. The FLS- Ca^{2+} homeostasis system is a primordial candidate to play a role in this process.

THE POSSIBILITY THAT SOME SERCA PUMP ISOFORMS ACT AS PROMISCUOUS RECEPTORS

In endocrinology, the consensus is that a ligand should bind with high affinity and specificity to a receptor. This holds true for plasma membrane receptors of the G-protein coupled type (GPCRs), and probably for intranuclear receptors as well as. We doubt that it also applies to lipophilic steroids and other lipophilic signaling substances like JH.

The question is raised whether the end of the juvenile state is caused by increasing concentrations of steroid hormones in the intracellular membrane continuum of cells that compete with FLS for binding to the receptor site on (some isoforms of) the SERCAs? In other words, does the endocrinological explanation for ending the juvenile state reside in the fact that the receptor site for farnesol/FLS on the SERCAs is (very?) promiscuous?

A major argument in favor of this view is that about 4,200 compounds have been synthesized or extracted from natural sources that are all active in bioassays for JH [=Juvenile Hormone Analogs or JHAs; (8)]. Some are farnesol-like; others have very different chemical structures. The most bioactive ones are synthetic peptides that are selectively active in insect species belonging to the Pyrrhocoridae. There are also anti-juvenile compounds (32), e.g.,

some benzodioxoles that act as non-mutagenic insect chemosterilants. It is improbable that all these compounds would bind to a different nuclear receptor but nevertheless generate similar morphological and physiological effects. The reason is that the number of nuclear receptors is estimated to be around 1,000. We think that it is more probable that many/most JHAs and sex steroids bind with a different affinity to a promiscuous receptor residing in the SER/RER. For other arguments, both in favor and against this view see De Loof (20).

Males and females do not have different sex steroids. In vertebrates both have estrogens and androgens. In insects both have 20-OH-ecdysone (=estrogen counterpart) and ecdysone (=testosterone counterpart) (20, 33). This raises the question which signaling system can handle a balance of hormones? We think that a promiscuous receptor can do so, although other possibilities cannot be excluded (20). Such a system takes into account not only the affinity of the ligand but the concentration of other competing ligands as well. A physiological effect is generated when the balance is “right.”

SEVERE OBESITY IN INSECTS NEAR THE END OF THE JUVENILE STATE: A CAUSAL LINK WITH FLS/Ca²⁺-HOMEOSTASIS?

The juvenile state is characterized by fast growth. This requires that tissues deposit more proteins than lipids. At the end of the end of the juvenile state, this situation is reversed in most species. Storing more lipids is often considered as beneficial “because accumulating nutrient reserves will facilitate the growth of reproductive organs and production of gametes, etc.” This reasoning is incorrect because it is teleological. Individual tissues, such as adipose tissue, do not plan for the future at all. They do not deposit lipids and glycogen because that would be beneficial for the fitness of the whole organism, but because a signal inside the adipose tissue is causal to the increased lipid production and accumulation. This raises the question as to the nature of that signal. Systems in which such drastic accumulation occurs in a short time are good experimental models, e.g., during pre-metamorphosis and during pre-diapause in insects.

The literature on obesity in vertebrates, in particular in humans is truly vast. Indeed, a Pubmed search with “obesity” as query (05/10/2014) yielded 205,506 references. Yet, one cannot escape the impression that, despite the enormous research effort, there is still no consensus about the primordial cause(s). Maybe something has been overlooked. Could it be FLS/Ca²⁺-homeostasis?

The query “obesity and calcium” yielded 3,155 references while “obesity and farnesol” resulted in only 3 refs of which one was quite interesting. Duncan and Archer (34) administered farnesol to rats. They found that oral administration lowers serum triglyceride levels. They think that the effect is mediated by the downregulation of retinoid X receptor beta. These authors assumed that farnesol, as a dietary component, could play a role in lipogenesis and fatty acid oxidation, both impaired in obesity. The possibility that farnesol/FLS are secreted into the bloodstream from tissues that produce large amounts of farnesol/FLS and cholesterol was not considered. Our data in **Table 1** show that one has to consider the possibility that FLS present in various tissues and in blood are synthesized by the body itself.

METAMORPHOSIS

We think that the FLS-lipogenesis connection deserves an in depth analysis. For this view, we focus on the situation in holometabolous insects like flies, beetles, butterflies, moths, etc. They differ from hemimetabolous ones like cockroaches, crickets, etc. in that they undergo a complete metamorphosis. The larval stage molts into a pupa and next into an adult. Prior to entering the pupal stage, the pre-metamorphic larvae undergo drastic changes. One in particular is that the penultimate and early last larval instars eat voraciously. A result is that their fat body accumulates huge amounts of lipids, glycogen, and some proteins as well with a fast gain in weight as a result. This eating comes to a complete stop during the last larval instar. Next they enter the wandering stage during which they leave the food in search for a suitable place to molt into a pupa. They empty their gut, by vomiting and/or defecation. In some species like, e.g., silk moths in which the silk glands are modified salivary glands, or *Drosophila* that uses secretions from their salivary glands as a glue to adhere to a substrate, these parts of the alimentary canal are also emptied. The larvae will become more and more immobile: they give the impression to enter in a coma-like state which lasts for some time. Meanwhile, the body is drastically reshaped. One of the changes is that the fat body is almost completely lysed. Like most other tissues that actively secrete proteins during the larval stage (2), they are replaced by adult-type tissues. Also the pupal cuticle is replaced by an adult one. At eclosion, a drastically “novel” organism makes its entrance into the world. Its larval morbid obesity is completely cured. Concurrently, the alimentary canal of the adult is drastically reshaped. Adults can still ingest food (not the case anymore in some species that do no longer have mouthparts), but the food they can handle can be totally different from what they ate as larvae (e.g., in flies and in butterflies and moths; some beetle species do not change their diet). One could say that they underwent a severe but efficient bypass of the stomach in combination with adaptation to a novel diet.

For physiologists and endocrinologists, the interesting question is: What causes this larval obesity and how is it cured? The key endocrinological event/change that takes place at the end of larval life is that the production of JH by the CA comes to a complete standstill. Also, all JH that circulates in the hemolymph is completely metabolized by specific esterases. Therefore, it is likely that an important cause of aggravating obesity is the drop to zero of the JH titer. This is evidenced by the fact that application of synthetic exogenous JH can overrule the morbid obesity syndrome. This treatment induces, in some species at least, a larval molt into a supernumerary instar (8).

PREPARING FOR DIAPAUSE

Accumulation of lipids in the fat body in the last larval instar is not just a matter of more lipids that are deposited. In the absence of JH, the whole physiology of the fat body changes. This has been shown long ago by De Loof and Lagasse (35). They studied the changes in ultrastructure of the fat body of adult Colorado potato beetles raised in two different photo-regimes. Beetles raised in long day conditions (more than 12 hr light per day) will reproduce. Their fat body cells are rich in RER (for the production of yolk protein precursors). Not much lipids or glycogen accumulate

and no protein vesicles. The whole fat body volume is small. At the height of vitellogenesis, it is so small that it is difficult to imagine that it could account for all yolk protein synthesis needed for the large number of eggs (up to about 50–60 per day) the females produce.

When raised in short day conditions (less than 12 hr light per day), reproduction is inhibited and the animals prepare for entering diapause. They start depositing large amounts of reserves in the fat body. Not only lipids and glycogen accumulate but also large amounts of proteins, packed in vesicles. Short day conditions inactivate the *corpora allata*. Thus, it is the absence of JH that causes all these changes. Similar changes take place in the fat body of last instar beetle larvae, a stage in which JH is also absent (unpublished results).

With the insights gained in unraveling the mode of action of absence of JH in pre-metamorphosis (2) the cell biological explanation for the cited effects is probably as follows. As already cited repeatedly, SERCA pumps have a binding site for sesquiterpenoids. As long as the JH titer is high, the SERCA pumps transport Ca^{2+} into the lumen of the RER which causes a secretion of proteins along with Ca^{2+} through the RER–Golgi system. The SERCAs in the SER also pump Ca^{2+} into the lumen of the SER. It is a general rule that SER membranes harbor enzymes for lipid- and steroid biosynthesis (25). As long as the $[\text{Ca}^{2+}]$ in the SERs lumen is high, these enzymes seem to be inhibited. The result is that no extreme accumulation of lipids in the fat body will occur. When the JH titer drops to zero, Ca^{2+} is no longer pumped into the lumina of both RER and SER. The classical Ca^{2+} -stores start releasing part of their stored Ca^{2+} . The RER can no longer secrete proteins out of the cell. They remain inside the cell in the form of protein vesicles. In the SER the inhibition of enzymes active in lipogenesis and ecdysteroid synthesis is lifted, resulting in the accumulation of lipid droplets in the fat body and a rising ecdysteroid titer in the hemolymph. All this probably corresponds to early events in the apoptosis pathway.

Thus, as long as the JH titer is high, the apoptosis pathway is inhibited. When the JH titer drops, the apoptosis pathway is induced, resulting in the temporary benefit that willy-nilly reserves are stored that will get a secondary role, beneficial at the organismal level, for remodeling tissues during metamorphosis (13).

We think that the initial steps, namely the roles of a high JH titer and of high farnesol concentrations are probably very similar in inducing obesity in both insects and vertebrates. Morbid lipid deposition in obese humans may reflect some malfunctioning of the FLS/ Ca^{2+} -ATPase system. A problem is that in vertebrates a situation, in which all FLS can be made totally absent, like this is the case in pre-metamorphosing holometabolous insects, never occurs in life. Whether it can be realized by RNAi is not known. In our opinion, the link FLS- Ca^{2+} -obesity deserves further exploration.

PERSPECTIVES FOR FUTURE RESEARCH

The outline of a practical method for analyzing the biosynthetic pathway of FLS (24), in combination with the awareness that this pathway is highly conserved in all eukaryotes, from yeast to mammals, arthropods, and nematodes, opens quite some perspectives for innovative research. It urges for evaluating whether, perhaps

this signaling pathway with a causal links to the universal Ca^{2+} -homeostasis system might play a key role in maintaining the juvenile state in vertebrates. We hypothesize that the comparison of the pathway in various tissues during successive developmental stages may yield indications whether changes might be causal to state change. Furthermore, a renewed interest in endogenous sesquiterpenoids could, perhaps, contribute to solving some standing problems in medicine.

1. The Hutchinson–Gilford progeria premature aging syndrome has a link to FLS, but its exact mode of action is only partially understood. This type of progeria is a lamin disease, a disease of the nuclear envelope. The cause of this genetic disorder is known. The gene LMNA encodes a protein named prelamin A. Through a process known as prenylation which is not only active in this syndrome, a farnesyl group gets attached to the carboxy-terminus of prelamin A. The farnesyl group allows prelamin to temporarily attach to the nuclear rim. Once the protein is attached, the farnesyl group is removed in normal persons/cells, while in progeria patients it remains attached. The result is that the no longer-farnesylated prelamin, which is now called lamin, does not remain anchored to the nuclear rim. The nucleus displays a normal shape. Failure to remove the farnesyl group permanently affixes the abnormal protein, now called progerin to the nuclear rim. This results in an abnormal shape of the nucleus² (36) (**Figure 1**).

It remains unclear how an abnormal shape of the nucleus can cause accelerated aging. There are numerous theories on the causes of aging; for a concise summary see De Loof et al. (19). But if the commonly held view that the nucleus is not an ionically isolated compartment is correct, the form of the nuclear envelope should not matter much. However, if the nucleus has the necessary tools to create its own nucleus-specific environment, a largely overlooked additional level of control of gene expression by inorganic ions (37) emerges. Although the published experimental data are scarce, there is now enough evidence to conclude that the nuclear envelope harbors ion pumps and channels to create a specific intranuclear environment (38–40). The discovery of Ca^{2+} -sensitive transcription factors (41) is one of the arguments in favor of the view that the nucleus has its own specific Ca^{2+} -homeostasis system (20).

2. With respect to research focused on aging, we think that in the list of nine hall marks of aging (42), two key causes of aging are missing, namely fading cellular electricity (19) and damage due to long lasting excess Ca^{2+} with its causal link to the still poorly understood role of endogenous sesquiterpenoids (2).
3. Alzheimer's disease: for the moment being (2014) there seems to be some disappointment among researchers that the enormous research effort that has been invested in searching for methods to prevent and cure this very severe disease did not yet yield promising results. A major problem is that the symptoms in humans sometimes become visible about 20 years after the disease started to develop. We think that, perhaps, unraveling the normal mechanisms of being a juvenile may contribute to

²<http://en.wikipedia.org/wiki/Progeria>

a better understanding of what can go wrong after the juvenile state ends.

4. Ca^{2+} -Homeostasis and the skeleton.

In juveniles, the skeleton of girls is on the average lighter than that of boys. Which elements of the Ca^{2+} -homeostasis system are involved and what exactly the role of sex steroids is, remains poorly understood. Osteoporosis, a multifaceted syndrome can become problematic in later life, in particular (but not exclusively) in postmenopausal women. Apparently the system that makes that females extrude more Ca^{2+} from their body than males (20) is already operational from very early in development on until very late in life. Whether manipulating the mevalonate-farnesol pathway may reduce the Ca^{2+} -loss may be worth investigating.

FINAL CONCLUSION

The evolutionarily ancient and very well conserved biosynthetic pathway leading from mevalonate to farnesol and derivatives is well documented in insects, but in vertebrate endocrinology and physiology hardly any attention has been paid to it. Yet, we think that not only in insects but in vertebrates as well endogenous FLS play a key role in many aspects of development and physiology. Our ideas can be summarized in the – admittedly simplistic – one-liner: “Farnesol/FLS help to smell well, to keep $[\text{Ca}^{2+}]_i$ low and to stay young and slim.”

DISCUSSION

Time has come to abandon the commonly held view that in vertebrates the juvenile state is automated, meaning that it is purely genetically controlled and that, unlike in insects no specific functional counterpart of a “JH-type signaling substance” is needed. All physiological processes and states of all organisms are controlled one way or another, not necessarily all by hormones.

The theory of evolution states that all contemporary organisms are the progeny of LUCA, the Last Universal Common Ancestor. One should not too readily assume that LUCA was a primitive organism. The environment in which LUCA lived was most probably hostile in several ways. The concentration of Ca^{2+} in the watery environment had probably risen far above the concentration that can hardly be tolerated in the cytosol of contemporary cells, namely about 100 nM. Above that threshold, Ca^{2+} starts changing the conformation and activity of some macromolecules, in particular proteins and chromatin. In other words it becomes toxic. Exactly this property makes that it can act as a secondary messenger in cells, on condition that its concentration rise does not last long. When the rise is more substantial and lasts longer, excess Ca^{2+} can become so toxic that the apoptosis-programmed cell death pathway is induced (21). This could be worded as “the Ca^{2+} -paradox: intracellularly toxic at low concentrations, but at the organismal level often beneficial at very high concentrations.” This implies that Ca^{2+} -homeostasis was already of outmost importance a couple of billion years ago. At least in some species, its underlying mechanisms must have been shaped to near perfection long ago; otherwise “life” would have become extinct. The fact that the structure of Ca^{2+} -ATPases of plants, insects, mammals, etc. resembles each other so very well, indicates that the Ca^{2+} -ATPases they inherited from LUCA were already shaped to

near perfection, and could not undergo substantial changes without becoming less efficient. Another implication is that the way in which the Ca^{2+} -homeostasis system is regulated must be very well conserved as well.

All this may sound very logical but it raises the question: What does Ca^{2+} -homeostasis and the way it is controlled have to do with being in the juvenile state? It took a touch of serendipity to find the link. It was first found in insects (20), in the following way. Already in Kopeć (43), a Polish biologist and pioneer in insect endocrinology, described that the brain of the moth *Lymantria dispar* is needed for normal development (43). Later, it was found that a pair of tiny glands, the *corpora allata*, which form part of the whole “brain complex,” secrete the hormone “JH.” When these glands are extirpated, precocious metamorphosis is initiated. As cited before, JH turned out to be a simple ester(s) of farnesol. The exact mode of action of JH remained ambiguous until very recently when De Loof et al. (2) re-analyzed the published data. They observed that it had been overlooked that in holometabolous insects the drop to zero of JH titer caused apoptosis in those tissues that had been actively secreting proteins during larval life. The calcium-induced apoptosis paradigm of Orrenius et al. (21) gave the hint that, perhaps, the presence of a high JH titer is required to keep the $[\text{Ca}^{2+}]_i$ in the cytosol low. In other words, the juvenile state of insects requires that $[\text{Ca}^{2+}]_i$ is kept low, therefore that the Ca^{2+} -pumps keep pumping. The lucky touch of serendipity came when De Loof et al. (2) started searching for known blockers of the Ca^{2+} -pump. Evidently, the well-known SERCA blocker thapsigargin showed up. A search for the chemical structure of thapsigargin showed that thapsigargin is, like farnesol and JH, a sesquiterpenoid. Because administration of thapsigargin induces apoptosis in both vertebrates and invertebrates, the conclusion was reached that at least the SERCA pump has a receptor site for sesquiterpenoids. Thus this type of Ca^{2+} -pump (to our knowledge no data on PMCA in the literature) is subject to regulation by endogenous sesquiterpenoids. If the receptor site is promiscuous as suggested by De Loof (20), other lipophilic signaling molecules may compete, in a concentration- and affinity-dependent way in binding to this overlooked type of receptor.

The next step was to evaluate whether the basics of the system that insects use to maintain their juvenile state might also apply to vertebrates. The rationale of this idea was that the control mechanisms for being in the juvenile state have probably been very well conserved since LUCA. This is apparent from the low degree of variability in Ca^{2+} -homeostasis systems among plants, vertebrates, invertebrates, etc. The major differences in the role of a FLS among animals are twofold. In insects and nematodes the JH/FLS reaches all cells of the body as a hormone, and, in insects at least its titer in the hemolymph can fall to zero prior to metamorphosis. In vertebrates FLS are synthesized in all cells and throughout life. This corresponds to a mode of action as an “inbrome.” The final effect on Ca^{2+} -homeostasis is probably very similar in all eukaryotes.

The concept we outlined here introduces a novel way of thinking, not only on how being a juvenile is regulated, but also on quite some other aspects of evo-devo. It emphasizes that in developmental processes researchers should leave more room for the controlling role of particular inorganic ions, especially Ca^{2+} , as well as to the electrical aspects of cell physiology (37, 44, 45). These

have come into tribulation by the enormous success of molecular biological techniques that gradually stressed ever more genes and transcription factors.

We are very well aware that our concept is still in the hypothesis and theory phase, and that a lot of additional experimental work is needed before our concept will gain “more body.” The major breakthrough will probably come when the binding site of thapsigargin on the SERCA pump will be chemically defined. The next step would then be to determine the degree of promiscuity of that largely overlooked receptor site.

The advent of the novel method of Rivera-Perez et al. (24) for analyzing which endogenous FLS are synthesized in any tissue via the mevalonate pathway may open new avenues in the numerous fields in which Ca^{2+} -homeostasis plays a role, e.g., in metabolism and reproduction (46). The data shown in **Table 1** are preliminary and do not exclude that some compounds could have been ingested with the food, but they nevertheless show that FLS are (differentially) omnipresent in the body. The fact that the compounds listed in **Table 1** are the same as the ones present in the *corpora allata* of insects, the only tissue that makes them during the larval state, and in nematodes as well (unpublished results) indicates that the mevalonate-FLS biosynthetic pathway is probably as essential in vertebrates as it is in insects and probably all other eukaryotes.

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Reconstructing SALMFamide neuropeptide precursor evolution in the phylum Echinodermata: ophiuroid and crinoid sequence data provide new insights

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The SALMFamides are a family of neuropeptides that act as muscle relaxants in echinoderms. Analysis of genome/transcriptome sequence data from the sea urchin *Strongylocentrotus purpuratus* (Echinoidea), the sea cucumber *Apostichopus japonicus* (Holothuroidea), and the starfish *Patiria miniata* (Asteroidea) reveals that in each species there are two types of SALMFamide precursor: an L-type precursor comprising peptides with a C-terminal LxFamide-type motif and an F-type precursor solely or largely comprising peptides with a C-terminal FxFamide-type motif. Here, we have identified transcripts encoding SALMFamide precursors in the brittle star *Ophionotus victoriae* (Ophiuroidea) and the feather star *Antedon mediterranea* (Crinoidea). We have also identified SALMFamide precursors in other species belonging to each of the five echinoderm classes. As in *S. purpuratus*, *A. japonicus*, and *P. miniata*, in *O. victoriae* there is one L-type precursor and one F-type precursor. However, in *A. mediterranea* only a single SALMFamide precursor was found, comprising two peptides with a LxFamide-type motif, one with a FxFamide-type motif, five with a FxLamide-type motif, and four with a LxLamide-type motif. As crinoids are basal to the Echinozoa (Holothuroidea + Echinoidea) and Asterozoa (Asteroidea + Ophiuroidea) in echinoderm phylogeny, one model of SALMFamide precursor evolution would be that ancestrally there was a single SALMFamide gene encoding a variety of SALMFamides (as in crinoids), which duplicated in a common ancestor of the Echinozoa and Asterozoa and then specialized to encode L-type SALMFamides or F-type SALMFamides. Alternatively, a second SALMFamide precursor may remain to be discovered or may have been lost in crinoids. Further insights will be obtained if SALMFamide receptors are identified, which would provide a molecular basis for experimental analysis of the functional significance of the “cocktails” of SALMFamides that exist in echinoderms.

Keywords: neuropeptide, echinoderm, SALMFamide, evolution, ophiuroid, crinoid

INTRODUCTION

The SALMFamides are a family of neuropeptides that occur in species belonging to the phylum Echinodermata (e.g., starfish, sea cucumbers, and sea urchins) (1). The prototypes for this neuropeptide family were discovered in the starfish species *Asterias rubens* and *Asterias forbesi* and are known as SALMFamide-1 (S1) and SALMFamide-2 (S2) (2, 3). S1 was identified as a C-terminally amidated octapeptide with the amino acid sequence Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH₂ (GFNSALMFamide) and S2 was identified as a C-terminally amidated dodecapeptide with the amino acid sequence Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH₂ (SGPYSFNSGLTFamide). Both peptides have the C-terminal motif FNSxLxFamide (where x is variable), which suggested that S1 and S2 may have evolved as a consequence of gene duplication or intragenic DNA duplication. Immunocytochemical investigation of the expression of S1 and S2 in *A. rubens* revealed

widely distributed patterns of expression in the nervous system but in separate populations of neurons (4, 5). Furthermore, S1 and S2 are present in the innervation of neuromuscular organs and, consistent with this finding, both peptides cause relaxation of starfish cardiac stomach, tube foot, and apical muscle preparations *in vitro* (6–9).

Subsequent to the discovery of S1 and S2 in starfish, SALMFamide-type neuropeptides were identified in other echinoderms. Thus, SALMFamide-type peptides were isolated from two sea cucumber species – *Holothuria glaberrima* and *Apostichopus japonicus* – and, consistent with the actions of S1 and S2 in starfish, these peptides cause muscle relaxation in sea cucumbers (10–12). It appears, therefore, that the relaxing action of SALMFamides on echinoderm muscle may be a general property of this neuropeptide family (9). Discovery of SALMFamides in sea cucumbers also revealed structural heterogeneity in SALMFamides. Two

SALMFamides isolated from *Holothuria glaberrima* were identified as GFSKLYFamide and SGYSVLYFamide, which share with the starfish SALMFamides S1 and S2 the C-terminal motif SxLx-Famide (i.e., L-type SALMFamides) (11). However, two SALMFamides isolated from *Apostichopus japonicus* were identified as GYSPFMFamide and FKSPFMFamide, which have the C-terminal motif SxFxFamide (i.e., F-type SALMFamides) (12).

Sequencing of the genome and transcriptome of the sea urchin *Strongylocentrotus purpuratus* (class Echinoidea) provided the first insight into the genetic basis of SALMFamide-type neuropeptide diversity in an echinoderm species (13). Thus, in *S. purpuratus* there are two genes encoding SALMFamide precursor proteins: one gene encodes a precursor comprising one L-type SALMFamide and one L-type-like (SxLxFamide) SALMFamide (14) and the second gene encodes a precursor comprising seven F-type SALMFamides (15). However, a more complicated picture has emerged as sequence data from other echinoderms has become available. The Holothuroidea (sea cucumbers) are a sister group to the class Echinoidea and, as in *S. purpuratus*, analysis of transcriptome sequence data from *A. japonicus* revealed two SALMFamide precursor transcripts. One of the precursors comprises three L-type or L-type-like SALMFamides and is homologous to the L-type SALMFamide precursor in *S. purpuratus* that contains two L-type or L-type-like SALMFamides (16). The second SALMFamide precursor in *A. japonicus* is largely comprised of F-type or F-type-like SALMFamides (five in total) but interestingly, unlike the precursor that gives rise to F-type SALMFamides in *S. purpuratus*, it also contains three L-type/L-type-like SALMFamides (16, 17). Thus, the “cocktail” of SALMFamides in *A. japonicus* is more complex than in *S. purpuratus*. What is not clear from these data, however, is which condition is ancestral and which is derived and to address this issue sequence data from other echinoderms is required.

Recently, genome sequence data for the starfish *Patiria miniata* has been obtained and this has revealed a SALMFamide profile similar to the sea cucumber *A. japonicus*. Thus, in *P. miniata* one SALMFamide precursor is solely comprised of L-type SALMFamides, which include S1 and six other structurally related peptides (16). The other SALMFamide precursor in *P. miniata* is largely comprised of F-type or F-type-like SALMFamides (eight in total) but it also contains an S2-like peptide with an L-type C-terminal motif (16). These predicted *P. miniata* SALMFamide precursor sequences now require confirmation by transcript sequencing. Nevertheless, the data currently available suggest that the occurrence of a precursor comprising several F-type SALMFamides and one or more L-type SALMFamides may be the ancestral condition, with the absence of L-type SALMFamides in the sea urchin F-type SALMFamide precursor being a derived condition. However, there remains the possibility that the occurrence of L-type SALMFamides in the F-type SALMFamide precursor is a feature that has arisen independently in both the holothurian and asteroid lineages. To gain further insight on this issue it will be necessary to determine the sequences of SALMFamide precursors in species belonging to two other extant echinoderm classes: the Ophiuroidea (brittle stars) and the Crinoidea (feather stars and sea lilies). As a sister group to the Asterozoa (starfish), the Ophiuroidea could provide key insights on SALMFamide precursor evolution. Thus, if the F-type SALMFamide

precursor in brittle stars also contains L-type or L-type like SALMFamides, as in starfish and sea cucumbers, this would add weight to the notion that this is a feature that dates back to the common ancestor of the Asterozoa (Asteroidea + Ophiuroidea) and the Echinozoa (Holothuroidea + Echinoidea). The Crinoidea are basal to the Asterozoa and the Echinozoa (18) and determination of the sequences of SALMFamide precursors in species belonging to this class of echinoderms could provide insight into the ancestral condition in the common ancestor of all extant echinoderms.

Here, we have analysed transcriptome sequence data from the starfish *P. miniata*, which in combination with genome sequence data has enabled definitive determination of the sequences of SALMFamide precursors in this species. Importantly, these data have also enabled comparison of SALMFamide gene structure in an asterozoan species (*P. miniata*) and an echinozoan species (*S. purpuratus*). Furthermore, here we report the sequences of novel SALMFamide precursors that we have discovered by analysis of transcriptome sequence data from the ophiuroid *Ophionotus victoriae*, a brittle star species that has a circumpolar distribution around Antarctica, and the crinoid *Antedon mediterranea*, a feather star species that is (as its name implies) native to the Mediterranean Sea. Having identified SALMFamide precursors in single species from each of the five echinoderm classes, we investigated the generality of our findings by analysis of genome/transcriptome sequence data from other echinoderm species.

MATERIALS AND METHODS

TRANSCRIPTOME SEQUENCING OF THE STARFISH *PATIRIA MINIATA*

Patiria miniata transcriptome sequence was produced using RNA from several developmental stages from blastula to juveniles, including bipinnaria and brachiolaria larvae. Sequencing was carried out at Huntman Genome Center University of Utah using Illumina HiSeq 101 paired end sequencing. 172,091,442 paired end reads were lightly trimmed and adapter sequences removed using Trimmomatic (19) with the following parameters: ILLUMINACLIP:illuminaClipping.fa:2:40:15 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:25. Post-quality trimming was assessed using FastQC¹. High-quality, trimmed reads were assembled *de novo* using the Trinity suite of programs, with default parameters except min_kmer_cov = 2 (20). The assembly yielded 203,888 transcripts, representing 101,664 transcript groups (genes). The contig N50 was 1717 bp, the median contig length was 502 bp, and the total assembled bases were 194,282,131. If only the longest isoform in each transcript group was considered, then the contig N50 dropped to 1490 bp, the median contig length was 389 bp, and the total assembled bases were 81,494,371.

TRANSCRIPTOME SEQUENCING OF THE BRITTLE STAR *OPHIONOTUS VICTORIAE* AND THE FEATHER STAR *ANTEDON MEDITERRANEA*

Arms dissected from a single adult specimen of *O. victoriae* and arms dissected from a single adult specimen of *A. mediterranea* were used for RNA isolation (Total RNA Isolation System, Promega, Southampton, UK). Library preparation (TruSeqv2 kit, Illumina, Little Chesterford, Essex, UK) was performed at the

¹<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>

QMUL Genome Center and sequencing (Illumina HiSeq 2500 platform) was performed at the BRC Genomics Core Facility at Guy's and St Thomas' NHS Foundation Trust and King's College London.

Illumina HiSeq sequencing yielded 155931609 and 116089417 paired 101 bp long reads for *O. victoriae* and *A. mediterranea*, respectively. Raw sequence data were assembled using the de Bruijn graph assembler Short Oligonucleotide Analysis Package SOAPdenovo-Trans-31mer 1.03² with the Kmer value set to 31, employing use of a high performance computing system (Apocrita³). The *O. victoriae* assembly yielded 669,744 contigs, with a mean length of 243 bp and N50 of 297 bp, and 17,616 contigs were >1000 bp in length. 76% of the contigs were assembled within scaffolds with a mean length of 300 bp and 30,859 scaffolds were >1000 bp in length. The *A. mediterranea* Kmer 31 assembly yielded 675,534 contigs, with a mean length of 254 bp and N50 of 310 bp, and 16,755 contigs were >1000 bp in length. 82% of the contigs were assembled within scaffolds with a mean length of 301 bp and 26,884 scaffolds were >1000 bp in length.

BLAST ANALYSIS OF THE ASSEMBLED TRANSCRIPTOMES OF *P. MINIATA*, *O. VICTORIAE*, AND *A. MEDITERRANEA*

To enable identification of transcripts encoding SALMFamide precursors in *P. miniata*, *O. victoriae*, and *A. mediterranea*, the contig and scaffold datasets generated from assembly of Illumina HiSeq sequence data obtained for these species were set up for BLAST (Basic Local Alignment Search Tool) analysis using SequenceServer⁴. BLAST searches were performed using the protein sequences of known SALMFamide precursors from *S. purpuratus*, *A. japonicus*, and *P. miniata* as queries (16). In addition, the sequence data were analysed by BLAST using short SALMFamide-type neuropeptide sequences as queries, with the *E* value set to 1000.

CLONING AND SEQUENCING OF SALMFAMIDE PRECURSOR CDNAS FROM *O. VICTORIAE* AND *A. MEDITERRANEA*

Total RNA that had been generated for Illumina HiSeq sequencing (see above) was also used for cDNA synthesis (Quantitect Reverse Transcription Kit, QIAGEN, Manchester, UK). Full-length cDNAs of SALMFamide precursors, including 5' and 3' untranslated regions (UTR), were amplified through PCR (Phusion High-Fidelity PCR Master Mix, NEB, Hitchin, Hertfordshire, UK) using the oligos: 5'-GTGACATTACTACTCCTGAT-3'/5'-CAACAAGACAGACTAATGAC-3' (*O. victoriae* L-type SALMFamide precursor), 5'-GAAGTGGTTGCTAATACC-3'/5'-ACTTTAGTCCTCCGTAC-3' (*O. victoriae* F-type SALMFamide precursor), and 5'-ATACAACGGGATAGAGAG-3'/5'-ACACTCGGAAGTTGTCTA-3' (*A. mediterranea* SALMFamide precursor), designed using Primer3 software⁵. The PCR products were gel-extracted and purified (QIAquick Gel Extraction Kit, QIAGEN, Manchester, UK) before being blunt-end cloned into a pBluescript SKII (+) vector (Agilent Technologies, Stockport,

Cheshire, UK) cut with the *EcoRV*-HF restriction endonuclease (NEB, Hitchin, Hertfordshire, UK). The clones were then sequenced (Eurofins Genomics GmbH, Ebersberg, Germany) from the T7 and T3 sequencing primer sites.

IDENTIFICATION OF SALMFAMIDE PRECURSORS IN OTHER ECHINODERM SPECIES

Our efforts to determine the sequences of SALMFamide precursors in this study or in previous studies (16) have targeted a single species for each of the extant echinoderm classes: *S. purpuratus* (Echinoidea), *A. japonicus* (Holothuroidea), *P. miniata* (Asteroidea), *O. victoriae* (Ophiuroidea), and *A. mediterranea* (Crinoidea). To assess the generality of our findings, ideally transcriptome and/or genome sequence data from multiple species for each echinoderm class would be analysed. As a step toward this level of analysis, here we have analysed genome or transcriptome sequence data from at least one additional species from each of the five echinoderm classes.

To search for SALMFamide precursors in a second echinoid species, genome sequence data from the sea urchin *Lytechinus variegatus* was analysed using a BLAST facility⁶ made publicly available by Andy Cameron and colleagues at CalTech (USA). To search for SALMFamide precursors in species belonging to the four other echinoderm classes, transcriptome sequence data that have recently been obtained for an investigation of echinoderm phylogenetic relationships was analysed (21). The data analysed included transcriptome sequences from a holothurian species (*Leptosynapta tenuis*), an asteroid species (*Luidia senegalensis*), a crinoid species (*Aporometra wilsoni*), and 52 ophiuroid species (21).

RESULTS

DETERMINATION OF THE SEQUENCES OF TRANSCRIPTS ENCODING SALMFAMIDE PRECURSORS IN THE STARFISH *P. MINIATA*

Previously, analysis of *P. miniata* genome sequence data enabled identification of two genes encoding SALMFamide-type neuropeptides in this species. Firstly, a gene encoding seven L-type SALMFamides (L-type gene) and secondly a gene encoding eight F-type SALMFamides and one L-type SALMFamide (F-type gene) (16). However, the predicted gene products reported by Elphick et al. (16) have yet to be confirmed by transcript sequencing. Therefore, here we analysed *P. miniata* transcriptome sequence data to identify SALMFamide precursor transcripts.

BLAST analysis of *P. miniata* transcriptome data using the predicted 174-residue *P. miniata* L-type SALMFamide precursor sequence as a query identified a 1657 bp transcript (contig 378809) encoding a 212-residue protein (Figure 1A; Figure S1 in Supplementary Material). Residues 63–212 of this protein were found to be identical to residues 25–174 of the predicted 174-residue protein. However, the N-terminal region of the 212-residue protein (residues 1–62) did not share sequence identity with the N-terminal region of the predicted 174-residue protein (residues 1–24). Thus, the predicted 174-residue sequence of the L-type SALMFamide precursor in *P. miniata*, which was based on analysis of

²<http://soap.genomics.org.cn/SOAPdenovo-Trans.html>

³www.hpc.qmul.ac.uk

⁴<http://www.sequenceserver.com>

⁵<http://bioinfo.ut.ee/primer3-0.4.0/>

⁶<http://www.echinobase.org/Echinobase/Blast/LvBlast/blast.php>

genomic sequence data, may be partially incorrect. Furthermore, BLAST analysis of *P. miniata* genome sequence data revealed that residues 1–62 of the 212-residue protein are encoded by an exon located on scaffold JH775329.1 (34230 bp), whereas residues 63–212 are encoded by an exon located on scaffold JH770521.1 (or 1914; 55,595 bp). Thus, the presence of the two exons on different genomic scaffolds provided an explanation for why the first exon was not identified when analysing genomic sequence data. The putative exon encoding residues 1–24 of the predicted 174-residue L-type SALMFamide precursor was identified on the same scaffold

A *Patiria miniata* L-type SALMFamide precursor

MKLYPLLAVAVLVFAGPFRIIEAYSPFGYHRRALLGNVWRASDNRRAPASTPEEEA
NEQRMGTAKRPAGSPVPHSALTYGKRAEDTDAAVERRAFHSALPFGKRTAMDRRG
LHSALPFGKRDDEAEQDALMERRGFNLSALMFGKRIHTALPFGKRGYHSALPFGKRS
DEEGTAMERRGYHTGLPFGKRDGTDAAVSEILSQLRSED

B *Patiria miniata* F-type SALMFamide precursor

MLRLVALLGIALLLVCQSAGQDAANAEQEDFYPAERTNGFDNLKGRSDDDQHLHS
ATKRDVSDRQREIDLAAQPPFYPGRRTDVPGRPSPGFVFGKRGQFFLPYQYKRELD
EVNPSYSAKRDELDTGLEEELDASKRNGPYSMGSLRSLTFGKREDAWSPEKRALDF
RSYAFGKRALGSNFAFGKRGYSSPFDGKRAGLGSSFTFGKRGVPDVRFADEDALLQ
DNKRALGSSFSFGKRSGLSSFTFGKRAGER

FIGURE 1 | *Patiria miniata* SALMFamide precursors. (A) L-type SALMFamide precursor. (B) F-type SALMFamide precursor. The predicted signal peptide of the precursor proteins is shown in blue and the putative SALMFamide neuropeptides are shown in red, with C-terminal glycine residues that are potential substrates for amidation shown in orange. Putative cleavage sites are shown in green.

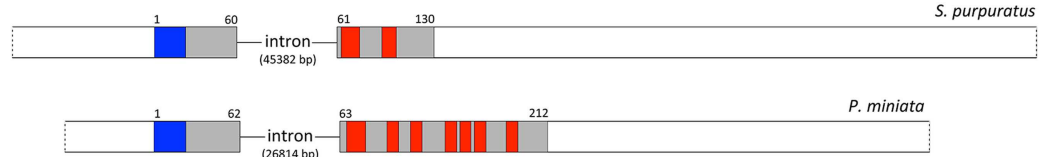
(JH770521.1) as the exon encoding the C-terminal region of the protein and was a plausible candidate exon because it encodes a polypeptide that has the expected properties for the N-terminal signal peptide region of a neuropeptide precursor. Transcriptome sequencing indicates that this prediction may have been wrong. However, there remains the possibility that this gene is subject to alternative splicing and transcripts encoding the predicted 174-residue L-type SALMFamide precursor occur naturally but are less abundant than transcripts encoding the 212-residue protein.

BLAST analysis using the predicted 258-residue *P. miniata* F-type SALMFamide precursor sequence as a query identified a 2041 base transcript (contig 387722) encoding a 258-residue protein that was identical to the query sequence (Figure 1B; Figure S2 in Supplementary Material). Thus, in this case analysis of transcriptome sequence data has provided confirmation of a sequence predicted from analysis of genome sequence data.

COMPARISON OF THE STRUCTURE OF SALMFAMIDE PRECURSOR GENES IN *S. PURPURATUS* AND *P. MINIATA*

Identification of transcripts encoding SALMFamide precursors in the starfish *P. miniata* (see above) has enabled analysis of the structure of the genes that encode these proteins and comparison with the structure of genes encoding SALMFamide precursors in the sea urchin *S. purpuratus*. Figure 2A shows a diagrammatic representation of the structure of genes encoding L-type SALMFamide precursors in *S. purpuratus* and *P. miniata* and Figure 2B shows a diagrammatic representation of the structure of genes encoding F-type SALMFamide precursors in *S. purpuratus* and *P. miniata*.

A L-type SALMFamide precursor genes



B F-type SALMFamide precursor genes

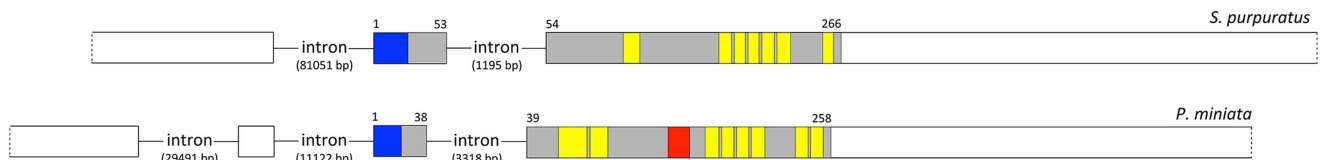


FIGURE 2 | Comparison of the structure of SALMFamide genes in the sea urchin *S. purpuratus* and the starfish *P. miniata*. (A) Diagrammatic representation of the structure of the L-type SALMFamide precursor gene in *S. purpuratus* and *P. miniata*. (B) Diagrammatic representation of the structure of the F-type SALMFamide precursor gene in *S. purpuratus* and *P. miniata*. Exons are drawn to scale as rectangles and introns are shown as lines but not to scale (intron lengths are shown in parentheses). Non-coding exons or regions of exons are shown in white; note that the 5' and 3' of the genes are shown as a dashed line because it is possible that additional non-coding bases are transcribed *in vivo* but are not present in transcripts that have been sequenced. The protein-coding exons or regions of exons are shaded (gray) or colored, with the N-terminal signal peptide shown in blue

and the predicted neuropeptide products of the precursors shown in red (L-type SALMFamides) or yellow (F-type SALMFamides). The numbers above the protein-coding exons are amino-acid residue positions in the protein products of the genes. A feature that appears to distinguish L-type and F-type SALMFamide genes is the structure of the first protein-coding exons. Thus, in the two L-type SALMFamide genes the first protein-coding exon also encodes a 5' non-coding region, whilst in the two F-type SALMFamide genes the first protein-coding exon does not have a 5' non-coding region. These differences in gene structure provide important evidence that the L-type SALMFamide precursor genes in *S. purpuratus* and *P. miniata* are orthologs and the F-type SALMFamide precursor genes in *S. purpuratus* and *P. miniata* are orthologs.

The L-type SALMFamide precursors in the two species have a similar gene structure comprising two exons. The first exon encodes a 5' non-coding region and the N-terminal region of the precursor proteins, including the signal peptide. The second exon encodes the C-terminal region of the precursor proteins (including the L-type SALMFamides) and a long 3' non-coding region.

The F-type SALMFamide precursors in the two species also have a similar gene structure but, importantly, the gene structure is distinctly different to the structure of the L-type SALMFamide genes. Thus, the F-type SALMFamide genes have one (*S. purpuratus*) or two (*P. miniata*) 5' non-coding exons followed by an exon that encodes the N-terminal region of the precursor protein, with the first codon of the exon encoding the starter methionine. This contrasts with L-type SALMFamide precursor genes (see above), where the exon encoding the N-terminal region of the precursor also encodes a 5' non-coding region. In common with L-type SALMFamide precursor genes, the C-terminal region of the F-type SALMFamide precursor proteins (which includes multiple copies of SALMFamide peptides) is encoded by a large exon with a long 3' non-coding region.

Thus, L-type SALMFamide precursor and F-type SALMFamide precursor genes are similar in that the N-terminal and C-terminal regions of the proteins are encoded by two exons. However, the presence (L-type) or absence (F-type) of a 5' non-coding region in the first protein-encoding exons is a distinguishing feature. This provides evidence that the L-type SALMFamide precursors in *S. purpuratus* and *P. miniata* are orthologous and F-type SALMFamide precursors in *S. purpuratus* and *P. miniata* are orthologous, which is an important finding because evidence for orthology based solely on comparison of protein sequences is not very strong, particularly for L-type SALMFamide precursors. Thus, whilst L-type SALMFamide precursors are characterized by the presence of peptides with a C-terminal SxLxLamide (L-type) motif, the number of copies of these peptides is very different in *P. miniata* (seven) and *S. purpuratus* (two). Indeed the presence of seven SALMFamides in the *P. miniata* L-type SALMFamide precursor makes this protein, at least superficially, more similar to F-type SALMFamide precursors in *P. miniata* and *S. purpuratus*, which contain nine and seven SALMFamides, respectively. In conclusion, therefore, the evidence of orthology provided by similarities/differences in gene structure provides an important basis for classifying SALMFamide genes in echinoderms as either L-type SALMFamide precursor genes or F-type SALMFamide precursor genes.

IDENTIFICATION OF AN L-TYPE SALMFAMIDE PRECURSOR IN THE OPHIUROID *O. VICTORIAE*

BLAST analysis of *O. victoriae* transcriptome sequence data revealed a 2422 bp contig (2059646) encoding a 169-residue protein comprising a predicted 20-residue N-terminal signal peptide and four putative SALMFamide neuropeptides bounded by monobasic or dibasic cleavage sites. A cDNA encoding this protein was cloned and sequenced, which confirmed the coding sequence predicted from assembled RNAseq data (Figure 3A; Figure S3 in Supplementary Material; GenBank accession number: KM979353). Analysis of the sequences of the four putative SALMFamide neuropeptides derived from this precursor

A *Ophionotus victoriae* L-type SALMFamide precursor

MRLQPLLVPFCICALVPFAATGTIPRRRSFGFEGANYNDVLVKDITQLEDENKEIDER
RSGRRNPSSLNSGLIFGKRFEAAEDFLNDDSRQINLVSRGSRLPFHSGMLMQCKRN
PLQDNLSVGRSRPQFHTGFMMCKRFTPEADDFDLEEFKRGKAGQRLRFSDGMLFGK

B *Ophionotus victoriae* F-type SALMFamide precursor

MARVRNILLILAAICCHATLSHAEDDETEELNHEQLVEFANKIMGQMKLLEYELGIQ
EHNDGQDMVKSLSKQAVRPGGGAPMNVFVKMSGFSFGKRDALVRRSAGATPSKL
AGFAFGKRGQPVKRSSDNEAEEQEKRGAMDAFAFGKPSGDPMSAFSFGKRNPMN
SLSAFAFGKAGMDPNLSNAFNGKRRDPLSAFSFGKRGMDLSAFNFGKRGDHL
AFSFGKGRNPMNGLSAFDGKRGMDAFAFGKREQEYNEEGAFDDEAEKRGYENGL
SGYAFGKRDITDDQLNHNDTLRTD

C *Antedon mediterranea* SALMFamide precursor

MFSQPLYLTLTWLFQHSLLAQGGTGDNIREGGVRYNRPHGGGVPSKANTSSSEPI
NNWIRALPVLHRLGYFGKRVPAANGYQLEDQFRDPAVAHLASKRNPALESEFMLGKRD
SFSSYMLGKRNRLSDMLGKRDPLSDMLGKRDPLSDMLGKRDPLSDMLGK
RDGFSDFTFGKRDALGDFMMGKREARLSDYIMGKRDPRISDFIMGRRELGENDVQR
HMGNNYDNKVEHEGKHVYVLSGDNREIEDNMNNVIYDDTDIPNQAESVSELQLEES
SSVGRKAKFQRPVYPNGKTPSQIWDTFGAGKRMSSVPDYDEEENVQTEKRSADP
KTSVRRFPFPAALHKGlyFGKRAATWADM

FIGURE 3 | *Ophionotus victoriae* and *Antedon mediterranea* SALMFamide precursors. (A) *O. victoriae* L-type SALMFamide precursor. (B) *O. victoriae* F-type SALMFamide precursor. (C) *A. mediterranea* SALMFamide precursor. The predicted signal peptide of the precursor proteins is shown in blue, putative neuropeptides derived from the precursors are shown in red with C-terminal glycine residues that likely substrates for amidation shown in orange. Putative cleavage sites are shown in green.

revealed that the first (i.e., N-terminally located) is a dodecapeptide with a canonical L-type SALMFamide motif (SxLxLamide) and the fourth (i.e., C-terminally located) is a putative 14-residue neuropeptide with an L-type-like SALMFamide motif (SxMxLamide). The intervening second putative neuropeptide is an 11-residue peptide with the C-terminal pentapeptide sequence SGLMQamide, which is L-type-like based on the presence of the leucine residue but is otherwise very unusual in having a C-terminal glutamine residue. The third neuropeptide is also a putative 11-residue peptide but with the C-terminal pentapeptide sequence TGFMMamide, which is F-type-like based on the presence of the phenylalanine residue but it has C-terminal methionine residue. Thus, this is predominantly a precursor of L-type or L-type-like SALMFamide precursor, homologous with L-type SALMFamide precursors that have been identified in other echinoderms. However, it is atypical in containing an F-type-like SALMFamide with a C-terminal methionine.

IDENTIFICATION OF AN F-TYPE SALMFAMIDE PRECURSOR IN THE OPHIUROID *O. VICTORIAE*

BLAST analysis of *O. victoriae* transcriptome sequence data revealed a 2343 bp scaffold (64,804) encoding a partial protein sequence comprising a predicted 23-residue N-terminal signal peptide and 11 putative SALMFamide neuropeptides bounded by dibasic cleavage sites. Because the protein-coding region of scaffold 64,804 was interrupted by a segment of unknown nucleotides it was necessary to clone and sequence a cDNA encoding this protein to determine its complete sequence. This revealed that it is a 310-residue protein comprising 12 putative SALMFamide

neuropeptides, 11 of them F-type SALMFamides, and 1 of them an L-type SALMFamide (**Figure 3B**; Figure S4 in Supplementary Material; GenBank accession number: KM979352). Thus, this precursor protein is similar to the F-type SALMFamide precursor in the starfish *P. miniata*, which comprises eight F-type or F-type-like SALMFamides and one L-type SALMFamide [**Figure 1**; (16)].

IDENTIFICATION OF A SALMFAMIDE PRECURSOR IN THE CRINOID *A. MEDITERRANEA*

BLAST analysis of *A. mediterranea* transcriptome sequence data identified a putative 1683 bp transcript encoding a SALMFamide precursor protein, which was assembled manually from three overlapping contigs (1781194, 446850, and 1694464) and then confirmed by cDNA cloning and sequencing (**Figure 3C**; Figure S5 in Supplementary Material; GenBank accession number: KM979351). The SALMFamide precursor is a 370-residue protein comprising a predicted 22-residue N-terminal signal peptide and 12 putative SALMFamide-type neuropeptides bounded by dibasic cleavage sites. Two of the putative peptides have a C-terminal LxFamide motif (i.e., L-type SALMFamide) and one of the putative peptides has a C-terminal FxFamide motif (i.e., F-type). However, the other nine putative peptides have a variety of C-terminal motifs that include FxLamide, FxMamide, YxLamide, YxMamide, and LxLamide. The motif F/YxL/Mamide has previously been referred to as “F-type-like” and the motif LxLamide has previously been referred to as “L-type-like” (16). However, as discussed below, discovery of the *A. mediterranea* SALMFamide precursor may require reclassification of SALMFamide neuropeptides in echinoderms.

IDENTIFICATION OF SALMFAMIDE PRECURSORS IN OTHER ECHINODERM SPECIES

Our findings from analysis of transcriptome or genome sequence data from other echinoderm species were consistent with those reported previously (16) or above, as described below and as illustrated in Figures S6–S10 in Supplementary Material.

An L-type SALMFamide precursor and an F-type SALMFamide precursor were identified in the sea urchin *Lytechinus variegatus* (class Echinoidea; Figure S6 in Supplementary Material), and as in *S. purpuratus*, the *L. variegatus* L-type SALMFamide precursor comprised two L-type SALMFamides (Figure S6A in Supplementary Material) and the *L. variegatus* F-type SALMFamide precursor comprised seven F-type SALMFamides (Figure S6B in Supplementary Material).

Interestingly, in the sea cucumber *Leptosynapta tenuis* (class Holothuroidea) two L-type SALMFamide precursors and one F-type SALMFamide precursor were identified (Figure S7 in Supplementary Material). Comparison of the sequences of the two L-type SALMFamide precursors in *L. tenuis* revealed high levels of sequence similarity (Figure S7C in Supplementary Material), indicating that these have arisen by gene duplication in this species or in a lineage that includes this species and other closely related holothurian species. As in the *A. japonicus* L-type SALMFamide precursor, both of the *L. tenuis* L-type SALMFamide precursors comprised three L-type or L-type-like SALMFamide neuropeptides (Figure S7A,B in Supplementary Material). The general characteristics of the F-type SALMFamide precursor in *L. tenuis* were

similar to the F-type SALMFamide precursor in *A. japonicus* in being largely comprised of F-type SALMFamides. However, the overall level of sequence identity was quite low and in the positions occupied by two L-type SALMFamides in *A. japonicus* there are F-type SALMFamides in *L. tenuis*. Analysis of a wider range of species will be required to determine which of these represents the ancestral/derived condition.

In the starfish *Luidia senegalensis* (class Asteroidea), an L-type SALMFamide precursor and an F-type SALMFamide precursor were identified (Figure S8 in Supplementary Material). The *L. senegalensis* L-type SALMFamide precursor comprises seven L-type SALMFamides, which share high levels of sequence similarity with the seven corresponding L-type SALMFamides in the *P. miniata* L-type SALMFamide precursor (Figure S8A in Supplementary Material). The *L. senegalensis* F-type SALMFamide precursor comprises eight SALMFamides, six of which are F-type or F-type-like, and two of which are L-type. By way of comparison, the *P. miniata* F-type SALMFamide precursor comprises nine SALMFamides, eight F-type, and one L-type (Figure S8B in Supplementary Material). Thus, whilst the overall organization of the F-type SALMFamide precursors in the two species is similar and clearly indicative of orthology, lineage specific gain/loss of peptides has occurred as well as conversion of peptides from F-type to L-type or vice versa. Analysis of a wider range of species will therefore be necessary to identify the ancestral characteristics of F-type SALMFamide precursors in asteroidea.

Recent molecular phylogenetic analysis of the class Ophiuroidea has identified three clades: clade A, clade B, and clade C (21). Here, an L-type SALMFamide precursor and an F-type SALMFamide precursor were identified in species belonging to each of these clades. In clade A, which includes *O. victoriae*, these were *Ophiomusium lymani* (Ophiolopidae), *Asteronyx loveni* (Asteronychidae), and *Asteroschema bidwillae* (Asterochematidae). In clade B *Clarkoma canaliculata* (Ophiocomidae) and in clade C *Ophiactis abyssicola* (Ophiactidae) and *Ophiothrix angulata* (Ophiotrichidae). The characteristics of the L-type SALMFamide and F-type SALMFamide precursor in these species were similar to those in *O. victoriae*, and this is illustrated using sequence data from *Ophiothrix angulata* as an example in Figure S9 in Supplementary Material. Thus, the L-type SALMFamide precursors in *O. victoriae* and *O. angulata* comprise four homologous peptides. However, as reported above for other classes, there is variable conservation of the sequences of peptides that occupy equivalent positions in the precursor proteins. For example, the C-terminal region of the first SALMFamide in the precursor is very similar in both species (LNSGLxFamide), whereas the third SALMFamide exhibits sequence divergence – TGFMMamide in *O. victoriae* and SAMLLamide in *O. angulata* (Figure S9A in Supplementary Material). The F-type SALMFamide precursor in *O. angulata* comprises 10 F-type SALMFamides and 1 L-type SALMFamide. This is similar to the F-type precursor in *O. victoriae*, which comprises 11 F-type SALMFamides and 1 L-type SALMFamide, with the L-type SALMFamide occupying the same position in both species (Figure S9B in Supplementary Material).

In the feather star *Apotometra wilsoni* (class Crinoidea), only a single SALMFamide precursor was identified, consistent with our findings from *A. mediterranea* (Figure S10 in Supplementary

Material). Comparison of the sequences of the SALMFamide precursor in *A. mediterranea* and *A. wilsoni* revealed high levels of similarity, with both precursors comprising 14 putative SALMFamide neuropeptides.

An example where there is sequence divergence is the seventh peptide, which has the C-terminal sequence LMLamide in *A. mediterranea* and FMLamide in *A. wilsoni*. However, peptides occupying equivalent positions in the two precursors are largely similar in their sequence characteristics.

DISCUSSION

Previously, analysis of genome/transcriptome sequence data has enabled identification of genes/transcripts encoding SALMFamide neuropeptide precursors in species from three of the five extant echinoderm classes: the sea urchin *S. purpuratus* (Echinoidea), the sea cucumber *A. japonicus* (Holothuroidea), and the starfish *P. miniata* (Asteroidea) (16). This has revealed that in each species there are two SALMFamide precursor genes: firstly, a gene that encodes peptides with a C-terminal LxFamide-type motif (L-type) and secondly, a gene that either only encodes peptides with a C-terminal FxFamide-type motif (F-type) (*S. purpuratus*) or encodes F-type SALMFamides plus one or more L-type SALMFamides (*A. japonicus*, *P. miniata*) (16). However, the *P. miniata* sequences were based on predictions made from genome sequence data, without supporting evidence from transcript sequences. Furthermore, a deeper understanding of SALMFamide precursor evolution in the phylum Echinodermata will require analysis of sequence data from at least one species from the two other extant echinoderm classes – the Ophiuroidea and Crinoidea.

Here, we have determined the sequences of transcripts encoding the L-type SALMFamide precursor and F-type SALMFamide precursor in the starfish *P. miniata*, which has enabled comparison of the structure of genes encoding these precursors with SALMFamide precursor genes in the sea urchin *S. purpuratus*. This has revealed that F-type SALMFamide precursor genes have one or two introns in the 5' non-coding region of the genes, whereas L-type SALMFamide genes do not have this feature. The overall level of sequence identity when comparing L-type SALMFamide precursors or F-type SALMFamide precursors protein sequences from different echinoderm classes is low [see Figures 5 and 6 in Ref. (16)]. Consequently, the categorization of SALMFamide precursors into two types (L-type and F-type) has hitherto been based upon the relative abundance of constituent peptides that have either an LxFamide motif or FxFamide motif. Obtaining evidence of orthology based on gene structure now provides an important additional criterion for classification of SALMFamide precursors as either L-type or F-type.

The F-type SALMFamide precursors in *A. japonicus* (echinozoan clade) and *P. miniata* (asterozoan clade) are largely comprised of F-type SALMFamides but they also contain one or more L-type SALMFamides. This suggests that the occurrence of L-type SALMFamides in F-type precursors may be a characteristic of F-type precursors traceable to the common ancestor of the Asterozoa and Echinozoa, with the absence of L-type SALMFamides in the F-type precursors of sea urchins (Echinoidea, Echinozoa) presumably a derived feature. Here, we have obtained evidence in support of this hypothesis with the identification of a transcript encoding

a F-type precursor in the brittle star *O. victoriae* (Ophiuroidea, Asterozoa). Thus, in *O. victoriae* there is an F-type precursor that largely comprises F-type SALMFamides (11) but which also contains an L-type SALMFamide. Similarly, the F-type precursor in the brittle star *O. angulata* comprises 10 F-type SALMFamides and 1 L-type SALMFamide. By way of comparison, the L-type SALMFamide precursors in *O. victoriae* and *O. angulata* comprise four putative peptides, including two L-type SALMFamides and an unusual peptide with a C-terminal LxQamide motif. This latter feature is atypical of L-type precursors that have been identified in other echinoderms and therefore it appears to be derived characteristic of ophiuroids.

Analysis of transcriptome sequence data from the crinoid *A. mediterranea* revealed only a single SALMFamide-type precursor. A homolog of this precursor was found in the feather star *A. wilsoni* and, as in *A. mediterranea*, this was the only SALMFamide precursor identified in this species. Analysis of the sequences of the putative neuropeptides derived from the *A. mediterranea* precursor reveals that it comprises five peptides with a FxLamide-type motif and four peptides with a LxLamide-type motif but only two peptides with a LxFamide-type motif and one peptide with a FxFamide-type motif. Based upon these characteristics, the *A. mediterranea* SALMFamide precursor does not appear to be an L-type-like precursor, which are largely comprised of peptides with a LxFamide motif, or an F-type-like precursor, which are largely comprised of peptides with a FxFamide motif. Thus, discovery of SALMFamide precursors in crinoid species has broadened our perspective on the structural characteristics of SALMFamides.

Hitherto SALMFamides have been characterized as either L-type (LxFamide) or F-type (FxFamide). Peptides that deviate from these motifs have been identified in echinozoans and asterozoans; for example, one of the peptides in the *A. japonicus* L-type SALMFamide precursor has the C-terminal motif IxLamide, which previously was categorized as L-type-like. Similarly, one of the peptides in the *A. japonicus* F-type SALMFamide precursor has the C-terminal motif FxLamide, which was categorized as F-type-like. Our discovery that the crinoid SALMFamide precursor largely comprises peptides with a LxLamide motif or LxLamide-like motif (e.g., LxMamide) and peptides with a FxLamide motif has provided a basis for categorization of SALMFamides into four types: 1. FxFamide, 2. FxLamide or FxMamide, 3. LxFamide, and 4. LxLamide (Figure 4). However, we retain the use of the terms L-type (LxFamide) and F-type (FxFamide) because these are useful for categorization of SALMFamide peptides and SALMFamide precursors in echinozoans and asterozoans. Furthermore, identification of transcripts encoding SALMFamide precursors in species representing all five extant echinoderm classes, the feather stars *A. mediterranea* and *A. wilsoni* (Crinoidea), the brittle stars *O. victoriae* and *O. angulata* (Ophiuroidea), the starfishes *P. miniata* and *L. senegalensis* (Asteroidea), the sea cucumbers *A. japonicus* and *L. tenuis* (Holothuroidea), and the sea urchins *S. purpuratus* and *L. variegatus* (Echinoidea), provides a basis for formulation of hypotheses on the evolution of these proteins.

Because crinoids are basal to the Echinozoa (Echinoidea and Holothuroidea) and the Asterozoa (Asteroidea and Ophiuroidea) in echinoderm phylogeny, one model of SALMFamide precursor

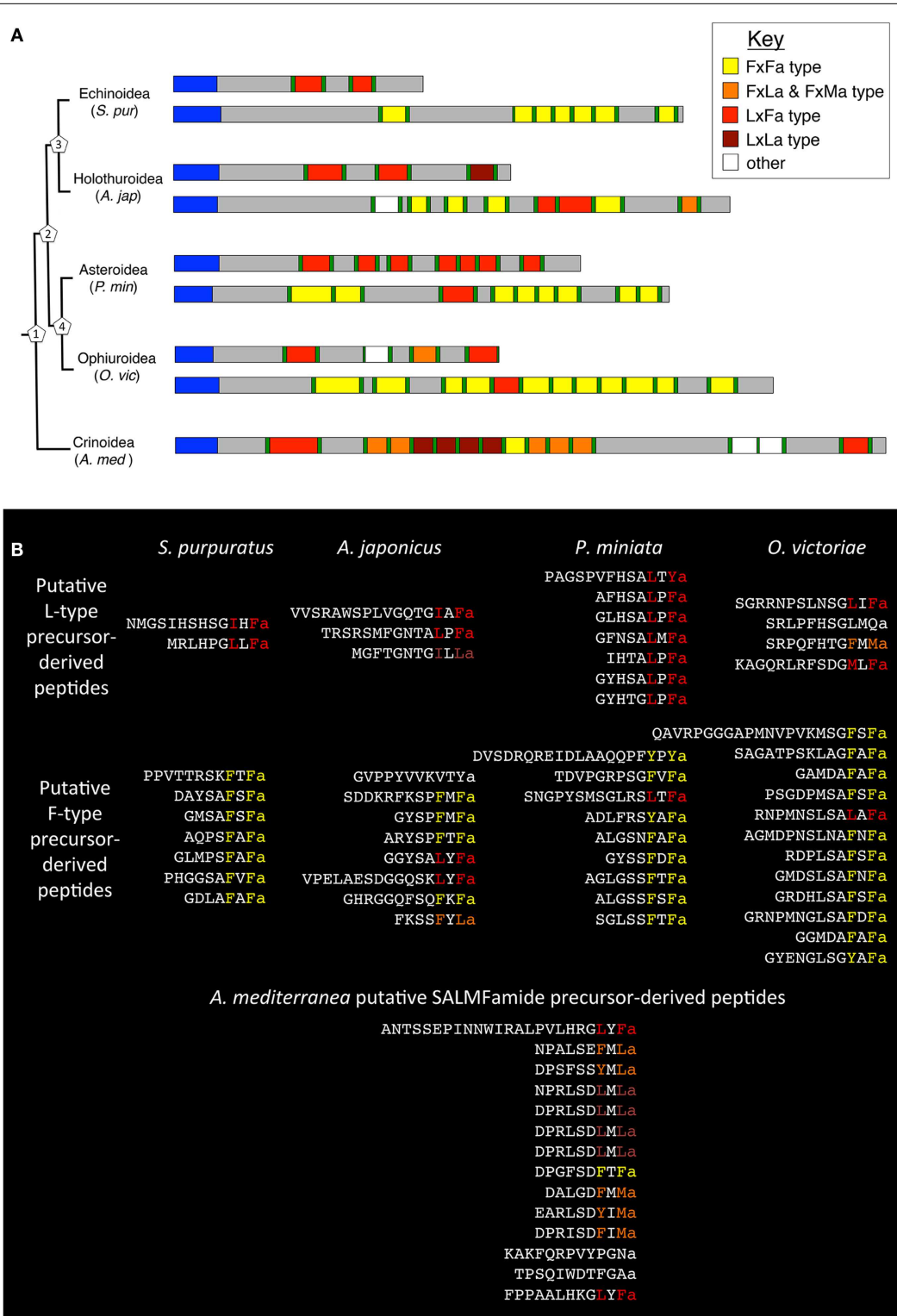


FIGURE 4 | The occurrence and properties of SALMFamide precursors (A) and putative SALMFamide peptides (B) in species representing each of the five extant echinoderm classes. (A) SALMFamide precursors are shown in a phylogenetic diagram according to the phylogeny of Telford et al. (18) and O'Hara et al. (21), with crinoids basal to the Echinozoa (Holothuroidea and Echinozoidea) and the Asterozoa (Asteroidea + Ophiuroidea). The estimated

divergence times for the nodes (labeled with numbers in pentagons) according to O'Hara et al. (21) are: 1. 501-542 Ma, 2. 482-421 Ma, 3. 464-485 Ma, and 4. at least 479 Ma. *S. pur* is the sea urchin *Strongylocentrotus purpuratus* (Echinozoidea), *A. jap* is the sea cucumber *Apostichopus japonicus* (Holothuroidea), *P. min* is the starfish *Patiria miniata* (Asteroidea),

(Continued)

FIGURE 4 | Continued

O. vic is the brittle star *Ophionotus victoriae* (Ophiuroidea), and *A. med* is the feather star *Antedon mediterranea* (Crinoidea). Signal peptides are shown in blue and dibasic or monobasic cleavage sites are shown in green. L-type SALMFamides with a C-terminal LxFamide motif or with an L-type-like motif (e.g., LxFamide) are shown in red. F-type SALMFamides with a FxFamide motif or with an F-type-like motif (e.g., YxFamide) are shown in yellow. SALMFamides with a FxLamide-type motif are shown in orange and SALMFamides with a LxLamide motif are shown in dark red. Peptides that do not conform with any of the four color-coded categories are shown in white (e.g., GVPPYVVKVTYamide in *A. japonicus* and SRLPFHSGLMQamide in *O. victoriae*). The diagram shows how in a

presumed ancestral-type precursor in crinoids the majority of the putative peptides have a FxLamide-type motif or a LxLamide-type motif and there is only one L-type SALMFamide and one F-type SALMFamide. However, as a consequence of specialization following a presumed duplication of the ancestral-type gene in a common ancestor of the Echinozoa and Asterozoa, two types of SALMFamide precursor have evolved: one that is predominantly comprised of L-type SALMFamides (red) and another that is exclusively or predominantly comprised of F-type SALMFamides (yellow). **(B)** C-terminal alignments of SALMFamide neuropeptides derived from the precursor proteins shown in **(A)**. The C-terminal regions of each peptide are color-coded according to the key shown in **(A)**.

evolution in the phylum Echinodermata is that ancestrally there was a single SALMFamide gene encoding a variety of SALMFamides (as in *A. mediterranea* and *A. wilsoni*), which duplicated in a common ancestor of the Echinozoa and Asterozoa. Then one of the duplicated genes specialized to encode L-type SALMFamides and the other specialized to exclusively or predominantly encode F-type SALMFamides (as in Echinozoa and Asterozoa). In the context of this evolutionary scenario, it is of interest to compare the structural features of SALMFamide precursors in the five echinoderm classes, using the precursors identified in *S. purpuratus*, *A. japonicus*, *P. miniata*, *O. victoriae*, and *A. mediterranea* as examples (see **Figure 4A**). Starting with the single SALMFamide precursor identified in the crinoid *A. mediterranea* and with reference to the key, which shows the color coding for the different types of SALMFamides, the predominance of peptides with a FxLamide-type or FxMamide-type motif (orange) or a LxLamide-type motif (dark red) is apparent, whilst the prototypical L-type SALMFamides (LxFamide; red) and F-type SALMFamides (FxFamide; yellow) are minor components. What cannot be deduced from this single precursor in an extant crinoid is whether or not this precursor structure is representative of an ancestral-type SALMFamide precursor that would have existed in extinct crinoid species ~550 million years ago, before the emergence of echinozoan and asterozoan echinoderms (18, 21, 22). In the lineage leading to extant feather stars (order Comatulidina; infraorder Comatulidia) such as *A. mediterranea*, intragenic duplication events may have resulted in expansion of segments of DNA encoding some SALMFamide neuropeptide types, whilst mutational changes or deletion events may have resulted in loss of other SALMFamide neuropeptide types. Further insight on this issue may be obtained if SALMFamide precursor transcript/gene sequences are obtained in the future with broader taxonomic sampling of extant crinoids. Nevertheless, we speculate that the occurrence of multiple types of SALMFamides (FxFamide-type, FxLamide-type or FxMamide-type, LxFamide-type, and LxLamide-type) in the *A. mediterranea* SALMFamide precursor is reflective of the ancestral condition in extinct crinoids that predated the emergence of asterozoans and echinozoans, although of course the precise number of copies of each type may be variable between extant crinoid species.

If the *A. mediterranea* precursor is representative of an ancestral-type SALMFamide precursor in being comprised of a variety of SALMFamides that have C-terminal FxFamide-type, FxLamide-type or FxMamide-type, LxFamide-type, and LxLamide-type motifs, then the occurrence of SALMFamide

precursors in asterozoans and echinozoans that are exclusively or predominantly comprised of peptides with a LxFamide-type motif (L-type precursor) or FxFamide-type motif (F-type precursor) is interesting from both an evolutionary and functional perspective. It suggests that following the putative duplication of a gene encoding an ancestral-type SALMFamide precursor in a common ancestor of echinozoans and asterozoans, there was specialization toward on the one hand a precursor of L-type peptides (LxFamide) and on the other hand a precursor of F-type peptides (FxFamide). Conversely, peptides with a LxLamide-type motif, FxLamide-type motif, or FxMamide-type motif that predominate in the *A. mediterranea* precursor are either absent or are minor components of SALMFamide precursors in echinozoans and asterozoans (see **Figure 4B**). This specialization of SALMFamide precursors as sources of either L-type or F-type SALMFamide neuropeptides would presumably reflect acquisition of distinct physiological roles. Therefore, it will be of interest to compare the patterns of expression of L-type precursors and F-type precursors and the pharmacological actions of their constituent neuropeptides in echinozoan and asterozoan species.

An alternative hypothesis to that outlined above is that a second SALMFamide-type precursor remains to be identified in crinoid species and that it remains undiscovered because of sequence divergence or incomplete transcriptome data. Another scenario is that two SALMFamide-type precursors existed ancestrally in crinoids but there has been loss of one precursor in a lineage that gave rise to extant feather star species such as *A. mediterranea* and *A. wilsoni*. If either of these scenarios is correct, then the question arises as to the relationship of the crinoid SALMFamide-type precursor identified here with the L-type and F-type SALMFamide precursors in asterozoa and echinozoa. Based on the number of constituent peptides, the crinoid SALMFamide-type precursor identified here is more similar to F-type SALMFamide precursors than L-type SALMFamide precursors. Further insights on the evolutionary relationships of SALMFamide neuropeptides and their precursors will be gained if the receptors that mediate their effects are identified.

AUTHOR CONTRIBUTIONS

This study was conceived and co-ordinated by Maurice R. Elphick. *P. miniata* transcriptome sequence data were obtained by Judith Levine and Christopher J. Lowe. *O. victoriae* and *A. mediterranea* transcriptome sequence data were obtained by Melody S. Clark, Maria Ina. Arnone, Dean C. Semmens, Liisa M. Blowes,

and Maurice R. Elphick. Analysis of *P. miniata*, *O. victoriae*, and *A. mediterranea* transcriptome sequence data was performed by Maurice R. Elphick, Dean C. Semmens, and Liisa M. Blowes. Cloning and sequencing of the *O. victoriae* and *A. mediterranea* SALMFamide precursor cDNAs was performed by Dean C. Semmens. The manuscript was written by Maurice R. Elphick and Dean C. Semmens, with all other authors editing or commenting on the final draft of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/Journal/10.3389/fendo.2015.00002/abstract>

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The “ram effect”: new insights into neural modulation of the gonadotropic axis by male odors and socio-sexual interactions

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Reproduction in mammals is controlled by the hypothalamo-pituitary-gonadal (HPG) axis under the influence of external and internal factors such as photoperiod, stress, nutrition, and social interactions. Sheep are seasonal breeders and stop mating when day length is increasing (anestrus). However, interactions with a sexually active ram during this period can override the steroid negative feedback responsible for the anoestrus state, stimulate luteinizing hormone (LH) secretion and eventually reinstate cyclicity. This is known as the “ram effect” and research into the mechanisms underlying it is shedding new light on HPG axis regulation. The first step in the ram effect is increased LH pulsatile secretion in anestrus ewes exposed to a sexually active male or only to its fleece, the latter finding indicating a “pheromone-like” effect. Estradiol secretion increases in all ewes and this eventually induces a LH surge and ovulation, just as during the breeding season. An exception is a minority of ewes that exhibit a precocious LH surge (within 4 h) with no prior increase in estradiol. The main olfactory system and the cortical nucleus of the amygdala are critical brain structures in mediating the ram effect since it is blocked by their inactivation. Sexual experience is also important since activation (increased c-fos expression) in these and other regions is greatly reduced in sexually naïve ewes. In adult ewes kisspeptin neurons in both arcuate and preoptic regions and some preoptic GnRH neurons are activated 2 h after exposure to a ram. Exposure to rams also activates noradrenergic neurons in the locus coeruleus and A1 nucleus and increased noradrenalin release occurs in the posterior preoptic area. Pharmacological modulation of this system modifies LH secretion in response to the male or his odor. Together these results show that the ram effect can be a fruitful model to promote both a better understanding of the neural and hormonal regulation of the HPG axis in general and also the specific mechanisms by which male cues can overcome negative steroid feedback and trigger LH release and ovulatory cycles.

Keywords: ram effect, odor, LH, estradiol, main olfactory system, experience, noradrenaline, kisspeptin

Introduction

Reproduction is essential for the survival and evolution of species and in most vertebrates it is controlled by similar networks of hormonal signals. The key regulator of the network is the hypothalamic neuropeptide, gonadotrophin releasing hormone (GnRH), which controls the release of the pituitary hormones, luteinizing hormone (LH), and follicle stimulating hormone (FSH). These latter hormones then stimulate the gonads to produce functional gametes and secrete estradiol, progesterone and testosterone that sustain reproductive function and auto-regulate the activity of the gonadotrophic axis by modulating the secretion of GnRH, LH, and FSH through positive and negative feedback systems. The mechanisms involved in the regulation of this network, often referred to as the hypothalamo-pituitary gonadal axis (HPG), have been the object of abundant research for several decades (Knobil, 1981; Karsch, 1984) but a central question which remained unresolved was how sex steroids modulate the activity of GnRH neurons while the latter lack receptors for steroids involved in feedback action (Herbison, 1998). However, the discovery of kisspeptin-containing neurons as being the most potent secretagogues of GnRH (Messenger et al., 2005), and the recent observation that, in mice, all kisspeptin neurons projecting on GnRH neurons have estradiol receptors (Kumar et al., 2015) make them the most probable target of steroid action (Clarkson and Herbison, 2009) and has opened up a new era in this field of research.

The HPG axis is also modulated by many internal and external factors such as nutrition, stress, immunological status, physical, and social environment (Signoret, 1980; Tomaszewska-Zaremba and Herman, 2009; Dobson et al., 2012; Follett, 2014; Roa and Tena-Sempere, 2014) but the mechanisms involved are largely unclear. The effects of the social environment are particularly intriguing because they are very diverse. In mammals they can inhibit reproduction such as in naked mole rat social groups in which reproduction is restricted to a few individuals (Goldman et al., 2006). This is also the case in marmoset family groups where the presence of the mother inhibits reproduction of her daughters (Abbott et al., 1981) or in mice where overcrowding can block reproduction (Whitten, 1959). In contrast, the presence of a sexual partner in many mammalian species can stimulate reproduction (Signoret, 1980; Vandenbergh, 2006) and may even be necessary for females to ovulate, such as in the cat, rabbit, and camel (Bakker and Baum, 2000). In sheep contact with a sexual partner has profound effects on reproductive events at all stages of reproductive life; it hastens puberty (Dyrmondsson and Lees, 1972), induces ovulation during seasonal anestrus (see review by Ungerfeld, 2007a,b) or lactational anestrus (Mauléon and Dauzier, 1965), and modifies the timing of the LH surge during the breeding season (Lindsay et al., 1975). The most spectacular and best known effect is the induction of ovulation in sexually quiescent females by exposure to a sexually active male, a phenomenon known as the “ram effect” in sheep (Martin et al., 1986; Ungerfeld, 2007a). This effect of a male has also been described in goats (Chemineau, 1983; Walkden-Brown et al., 1999) and in several wild ungulates (Skinner et al., 2002; Shipka et al., 2002).

The “ram effect” was discovered in 1944 (Underwood et al., 1944) but studies on the mechanisms involved only started in the 80's when reliable LH assays became readily available and when the sheep, because of their size, availability and economic importance became a widely used model for the study of the HPG axis (Karsch et al., 1997). The object of this review is to summarize what we have learned in the last few decades about the mechanisms involved in the “ram effect” and to discuss how this knowledge could help us to understand the regulation of the HPG axis more generally.

Description of the “Ram Effect”

Sheep are seasonal breeders. Ewes have regular 17 days oestrous cycles when day length is decreasing (the breeding season) and give birth in the spring when the environmental conditions are most favorable for the survival of their young. As day length increases ewes stop cycling (anestrus) but the introduction of a sexually active ram into a group of seasonally anoestrous animals will induce a pulse of LH (the short-term LH response **Figure 1A**) within minutes, whereas FSH does not undergo such a rapid change (Martin et al., 1980; Poindron et al., 1980). Exposure to a ram, or his odor, does not induce marked behavioral changes in ewes, but seems to focus their attention and induce urination (Gelez et al., 2004a). If contact with the male is maintained, the increase in pulsatile LH secretion initiates a sequence of physiological events that in some ewes will culminate in a LH surge 6–54 h later (Oldham et al., 1978; Chanvallon et al., 2011). Ovulation nearly always follows the LH surge (100% cases in Chanvallon et al., 2011; 97% in Scaramuzzi et al., 2014) but is described as “silent” because it is not associated with estrous behavior. Some females will then have a normal estrous cycle with a luteal phase lasting approximately 10 days and display estrous behavior 17–20 days after the introduction of rams (**Figure 1B**). In others, the corpus luteum from this first “silent” ovulation does not develop normally and regresses after a few days with a resultant short cycle; the ewe starts a new cycle but without a display of estrus (Oldham and Martin, 1978). Sexual behavior in these females only appears 22–28 days after the introduction of ram at the time of the third ovulation (**Figure 1B**). If ewes are mated at the time of the male induced estrus, a synchronized pattern of lambing occurs with two peaks 164 and 172 days afterwards. This singular pattern of births (**Figure 1C**) is the origin of the discovery of this phenomenon (Underwood et al., 1944) and could be used a convenient tool to identify those breeds responding to the “ram effect.”

Nearly all adult ewes have a short-term LH response after exposure to a sexually active ram during anestrus (93% in Chanvallon et al., 2011; 92% in Scaramuzzi et al., 2014). However, the intensity of this short term response varies and LH pulsatility after the “ram effect” is lower in ewes with low as opposed to high body condition (Scaramuzzi et al., 2014). An analysis of the LH response to a bolus of GnRH (75 ng) given to the same animals the day before they were exposed to the “ram effect” indicated that at least part of this variability was due to altered sensitivity of the pituitary. This was due to ewes with a low body condition having LH pulses of a lower amplitude in response to the GnRH

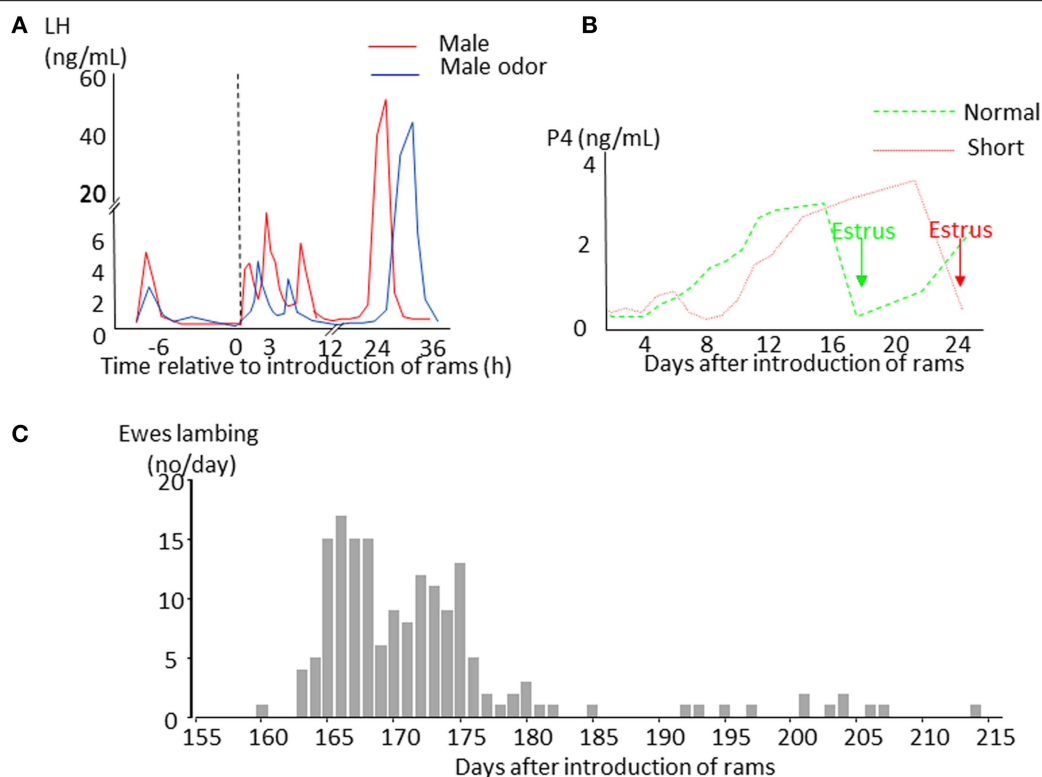


FIGURE 1 | Schematic representation of the effects of the introduction of sexually active rams to anestrus ewes. (A) Changes in LH plasma concentrations in ewes exposed to a sexually active ram (red line) or to its fleece (blue line). **(B)** Changes in progesterone plasma concentrations indicating formation of a corpus luteum. In ewes presenting a luteal phase lasting around 10 days (normal cycle green line), estrus (green arrow) is displayed 16–19 days after male introduction before the second ovulation. In ewes with a short luteal phase (short cycle red line),

estrus (red arrow) appears 21–26 days after ram introduction before the third ovulation. **(C)** Example of distribution of lambing in a flock of anestrus Mérinos d'Arles ewes exposed to the ram during the middle of anestrus. The ewes lambing during the first wave around 165 days after male introduction are those that first presented a normal cycle, those lambing in the second wave are those that first presented a short cycle. The ewes lambing later than 180 days are generally ones that did not become pregnant at their first mating.

bolus than those with a high body condition (Scaramuzzi et al., 2014).

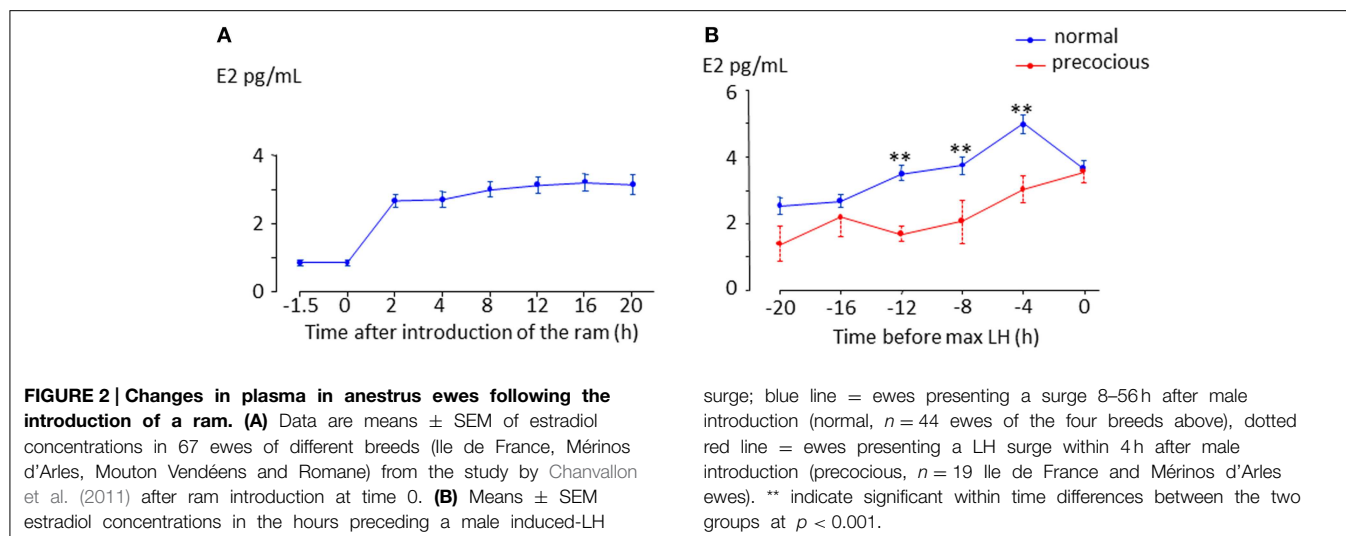
A short-term LH response to the presence of a ram is not restricted to the anestrus period and is observed in some cycling ewes during the luteal phase (Hawken et al., 2007; Chanvallon et al., 2010a) although less frequently than in anestrus ones. This is rather surprising because progesterone is known to have a strong inhibitory action on LH secretion (Goodman and Karsch, 1980; Goodman et al., 2002) and suggests that the network by which male cues stimulate LH secretion is at least partially different from that involved in ovarian steroid feedback. Interestingly the intensity of short-term LH secretion is a parameter that can predict the occurrence of ovulation since LH pulse frequency after the introduction of rams is higher in ewes that subsequently ovulate than in ones that do not (Chanvallon et al., 2011).

In contrast to the high incidence of short-term LH responses to cues from the ram, the frequency of actual LH surges and resultant ovulations is much more variable ranging from 0 to 100%. This is dependent upon many factors, but especially on the breed, age, experience, nutritional state of the animals and time of the year (Oldham et al., 1978; Chanvallon et al., 2010a,b, 2011; Johnson et al., 2011), suggesting that the induction of the

LH surge is the major cause of variability in response to the “ram effect.”

Role of Estradiol

In sheep, as in all mammalian species, the LH surge is stimulated by an increase in secretion of the hypothalamic neuropeptide, GnRH induced by an increase in circulating estradiol (Hauger et al., 1977; Karsch et al., 1979; Goodman, 1994) during the follicular phase. This phenomenon referred to as “estradiol positive feedback,” lasts 12–24 h depending on the breed (Land et al., 1976; Cahill et al., 1981; Ben Saïd et al., 2007). It is widely assumed that the LH surge following the “ram effect” is induced by the same “estradiol positive feedback” mechanism (Martin et al., 1986). However, partly because of the difficulty in measuring the very low concentrations of circulating estradiol present during anestrus there has been very little experimental support for this hypothesis (Knight et al., 1978; Johnson et al., 2011). In a recent study we showed that, in all ewes the introduction of rams is followed by an increase in the circulating concentration of estradiol (Figure 2A, Fabre-Nys et al., 2015). In most anestrus ewes, the LH surge induced by the “ram effect” was preceded



by increases in the circulating concentration of estradiol at least three-fold above the basal concentration for 14.5 ± 0.86 h (min 6 h; max 36 h, **Figure 2B**). Similar to the breeding season, the concentration of estradiol decreased at the time of the LH surge (**Figure 2B**).

The duration and pattern of these increases in estradiol concentrations varies among breeds. This variability in a highly seasonal breed, the Mouton Vendéen, is due to the low sensitivity of the ovary that releases very little amount of steroids in response to stimulation by a ram. The granulosa cells of these ewes in culture, also have a low response to *in vitro* stimulation by IGF-I and FSH and reduced expression of StAR (Fabre-Nys et al., 2015). In other breeds such as the Romane the frequency and latency of the LH surge is variable, although the quantities of estradiol secreted after the “ram effect” do not differ from those in breeds such as the Mérinos d'Arles and Ile de France that respond well to it. In these breeds the variability in response seems to be due to fluctuating sensitivity of the hypothalamo-hypophyseal complex to estradiol feedback.

In some ewes LH surges are induced immediately after exposure to a ram and these “precocious” LH surges are not preceded by increased concentrations of estradiol (Fabre-Nys et al., 2013; **Figure 2B**). Contrary to the spontaneous LH surges that occur in ewes during the breeding season, these “precocious” LH surges cannot be the result of classical “estradiol positive feedback.” Indeed the important question raised here is whether the mechanism of induction of these male-induced LH surges shares any similarities with that in spontaneous ovulators (Fabre-Nys et al., 2013).

The Stimuli Involved

The ram emits a considerable range of different sensory stimuli that could be responsible for evoking a reproductive neuroendocrine response in ewes, but olfactory stimuli clearly play a dominant role. Direct physical contact with a ram is not necessary (Watson and Radford, 1960) and a complete reproductive neuroendocrine response can be induced by exposure to ram

fleece (i.e., ram odor) alone (Knight and Lynch, 1980). However, fewer ewes ovulate when exposed only to ram odor (Pearce and Oldham, 1988) and additionally the frequency of short-term LH pulses is lower and they appear later (Gelez et al., 2004a). The active compounds involved are present in the fleece from all parts of the coat and in the anteorbital glands but are absent from urine (Cohen-Tannoudji et al., 1994). They are also androgen-dependant since ewes do not ovulate when exposed to a castrated ram (Fulkerson et al., 1981). A mixture of compounds is clearly involved because the biological activity of fleece requires the combined extracts of both the neutral and acidic fractions (Knight and Lynch, 1980; Cohen-Tannoudji et al., 1994). Those from the neutral fraction have been identified as 1,2-hexanedicandiol and 1,2-octanedicandiol, but those of the acidic fraction have not yet been identified (Cohen-Tannoudji et al., 1994).

The olfactory stimuli which generate the “ram effect” are not strictly species specific since hair from male goats stimulates LH pulsatile secretion (Over et al., 1990) and induces ovulation in ewes (Birch et al., 1989). A recent study showed that an acidic fraction of male goat hair that stimulated multiunit activity of the mediobasal hypothalamus of ovariectomized Shiba goats also stimulated pulsatile LH secretion in St Croix ewes (Ohara et al., 2014). The same group has also used this approach to show that 4-ethyltolanol is of key importance for activating the GnRH pulse generator in goats (Murata et al., 2014). This could therefore represent a very interesting way to approach the chemical identification of all the odorant compounds responsible for influencing the HPG axis.

However, the complete identification of all the active components of ram odor, might not be so simple a task because it appears that ewes need to “learn” to recognize them (see section below) and they can be trained to show an LH response to other odors. For example, ewes can show an increase in LH pulsatility in response to the odor of lavender if it has been associated with rams (Gelez et al., 2004a). This means that some odorant compounds may be common to both rams and bucks, some common to all rams but others may be specific to individual

rams. Sheep can distinguish between odor cues from different individuals (Baldwin and Meese, 1977) and the presence of such individual olfactory signatures is important for mate selection in rodents (Brennan and Kendrick, 2006). In sheep it may help ewes identify specific rams and explain the increased response to newly introduced, “novel” ones (Jorre de St. Jorre et al., 2012).

Non-olfactory stimuli are also involved since short-term LH responses can be observed in anosmic ewes exposed to sexually active rams (Cohen-Tannoudji et al., 1986) indicating that other sensory inputs can substitute for male odor. The intensity of male sexual behavior is also important with males exhibiting high libido appearing to be more effective than males with low libido in inducing ovulation in some studies (Signoret et al., 1982; Perkins and Fitzgerald, 1994), although not in others (Fisher et al., 1994). Visual cues alone from rams alone have very limited effects with exposure of ewes to projected images of rams only inducing small increases in LH secretion (Hawken et al., 2009a). Auditory cues also seem to have very limited effects (Hawken et al., 2009a) which is not that surprising because not all rams vocalize when courting ewes.

Factors Affecting Female Sensitivity to Male Stimuli

Breed and Time of Anestrus

The proportion of ewes ovulating in response to the “ram effect” varies with breed and time of year, the latter being highest in late anestrus (Ungerfeld, 2007b; Chanvallon et al., 2011). Sheep breeds also vary greatly in their sensitivity to photoperiodic cues (Malpaux, 2006).

Breeds are considered less seasonal if a high proportion of females are spontaneously cyclic during anestrus. This parameter is usually regarded as a sign of a “shallow” anestrus and is linked to a higher frequency of pulsatile LH secretion indicative of a lower response to the negative feedback of estradiol (Goodman et al., 1982). According to Lindsay and Signoret (1980) there is a positive correlation between the proportion of ewes in a flock that cycle spontaneously in anoestrus and that which ovulate after the “ram effect.” However, this theory has been challenged recently. In Limousine ewes, a moderately seasonal breed, Tournadre et al. (2002) found that the proportion of anestrus ewes ovulating was higher when there were fewer cyclic ewes in the flock. In another study comparing the responses of four French breeds of sheep Chanvallon et al. (2011) found that there was no link between the proportion of ewes ovulating after the “ram effect” and that of cyclic ewes present in the test flock. In some breeds, such as the Ile de France, ewes always show a high ovulatory response even if few females are cyclic in the flock, whereas the less seasonal Romane breed has highly variable ovulatory responses at the beginning of anestrus. This suggests the existence of a “sensitivity to socio-sexual stimulation” factor that in some conditions can override sensitivity to estradiol negative feedback.

Experience and Age

Young and sexually naïve ewes have a generally poorer ovarian response to the “ram effect” than adult, experienced ones (Oldham et al., 1984; Thimonier et al., 2000; Chanvallon et al., 2010a)

even though they show good short-term LH responses (Gelez et al., 2004a). Pre-exposure of young ewes to rams several months before the “ram effect” increased the proportion of ewes ovulating in one study (Murtagh et al., 1984) but failed to do so in another (Chanvallon et al., 2010b), and had no effect on the short-term LH response (Gelez et al., 2004a). Pre-exposure to rams however increased the short-term LH response when the stimulus at the time of the ram effect was the odor of the fleece of the male (Gelez et al., 2004a). This effect involved some “learning” of male characteristics. If during the pre-exposure, the male had been scented with lavender some ewes showed a short-term LH response to lavender, whereas ewes exposed to an unscented ram or to unfamiliar ewes scented with lavender did not (Gelez et al., 2004a). However, the conditions required for the ewes to learn the necessary male characteristics and the mechanisms involved are currently unclear (Chanvallon et al., 2010b). They are likely to occur at the level of brain areas known to undergo structural changes associated with olfactory learning involved in social recognition such as the hippocampus, amygdala and olfactory bulb (Brennan and Keverne, 1997; Sanchez-Andrade and Kendrick, 2009). Additionally, recent studies have reported plasticity changes in pituitary gonadotrope cells with increases in cell numbers and connectivity being found after puberty and lactation (Budry et al., 2011; Alim et al., 2012; Hodson et al., 2012). Thus plasticity changes in the pituitary itself might also contribute to the effect of sexual experience on the response to the “ram effect.” Clearly more research is needed to improve our understanding of these conditions and to provide more information on the circuits that enhance the ability of the HPG axis to respond to environmental cues. This would also provide information of potential economic value to farmers wanting to improve the responses of their flocks to the “ram effect.”

Stress

Stress can affect reproduction in many different ways (Rivier and Rivest, 1991; Ferin, 1993; Dobson et al., 2012). The abrupt change in the socio-sexual environment at the time of the “ram effect” may be stressful and especially so for young sexually naïve ewes. However, this area of research has received very little attention. To examine the potential role of socio-sexual stress on modifying responses to the “ram effect” both adult sexually experienced and young sexually naïve Merino ewes genetically selected for “calm” or “nervous” temperaments over 15 generations (Murphy et al., 1994) were compared after “ram effect” (Chanvallon et al., 2010a). The hypothesis was that having a “calm” temperament would help young sexually naïve ewes cope with this novel and potentially stressful situation and so improve their response to the “ram effect.” The neuroendocrine responses of all ewes were quantitatively the same although the adult experienced ewes had a faster neuro-endocrine response compared to young sexually naïve ewes independent of their temperament. Contrary to our expectation, fewer “calm” sexually naïve ewes ovulated after the ram effect; 18% compared 62% for “nervous” ewes and 100% in adults of either temperament. Being “nervous” seems to have helped the young sexually naïve ewes respond to the “ram effect,” possibly because they were more alert and attentive toward the male. In another experiment exposure to a series of different

acute stressors for the 2 days before and after the “ram effect” which increased cortisol levels decreased the proportion of young sexually naïve Ile de France ewes ovulating (Chanvallon et al., 2010b), although the short-term LH responses were not affected. The causes of these breed differences is unknown but in several species (rat, mice, pig, quail, human) the response to stress has a genetic component (Eley and Plomin, 1997; Mormede et al., 2011) and this may also apply to sheep.

Neural Circuitry Involved in the Ram Effect

Socio-sexual cues, similar to other factors that modulate reproduction (nutrition, stress, photoperiod), act on central nervous system networks that ultimately converge on the GnRH neurons and in this way modulate the activity of the HPG axis. According to Herbison (2006) the activity of each GnRH neuron could be affected by approximately 5 million neurons, so it seems a big challenge to understand how information derived from external cues such as the “ram effect” can have such a specific effect.

Most work has focussed on establishing the neural circuitry within the hypothalamus and preoptic regions which is critical for the male effect. In sheep, as in most mammals, GnRH is released in a pulsatile fashion with each pulse of GnRH inducing a pulse of LH which is the parameter generally analyzed in neuroendocrine studies. This pulsatile secretion of GnRH is regulated by gonadal steroids at the level of the mediobasal hypothalamus (Knobil, 1981; Karsch, 1984; Maeda et al., 2010; MBH) by mechanisms that are not yet clear (Tsutsumi and Webster, 2009). Studies from a Japanese group have correlated, multiunit electrical activity of neurons in the MBH with the LH response to male goat odor in ovariectomized females (Mori et al., 1991; Hamada et al., 1996; Murata et al., 2014) and this area of the brain is also thought to be central to the male effect in sheep (De Bond et al., 2013; Ohara et al., 2014).

By contrast, the surge mode of GnRH secretion that is responsible for the preovulatory LH surge has for a long time, been considered in rodents and sheep to emanate from the preoptic area (Herbison, 2006). In rodents only a subset of GnRH neurons located in the preoptic area around the organum vasculosum lateral terminalis (OVLt) are activated during the preovulatory LH surge (Lee et al., 1992). In sheep the GnRH neurons activated during the LH surge are not preferentially localized but are scattered throughout the entire distributed field of GnRH neurons (Moenter et al., 1993). Localized implantation of estradiol has also shown that the MBH is the critical area for estradiol positive feedback in the ewe (Blache et al., 1991; Caraty et al., 1998).

The precise neural networks linking the various cues associated with the “ram effect” to the GnRH neurons are not completely established. The focus to date has mainly been on how male odor cues can influence their activity since it is clear that olfactory cues are of the great importance (Swaney and Keverne, 2009; Baum and Cherry, 2015). However, there may be some species differences particular with regard to the involvement of the main and accessory olfactory systems.

In rodents, in which most studies have been carried out, the active chemosensory cues from sexual partners are mainly

detected and processed by the accessory olfactory system. Chemosensory cues from the male are detected by receptors in the vomeronasal organ and transmitted to the hypothalamus via the accessory olfactory bulb (AOB), with only one relay in the medial nucleus of the amygdala (Buck, 2000; Swann et al., 2009). Removal of the vomeronasal organs or lesioning of the accessory olfactory bulbs results in the disappearance of the effect of the partner (Beltramino and Taleisnik, 1983). However, this strict relationship between the accessory olfactory system and partner cues in rodents has been challenged by Yoon et al. (2005) who used transgenic mice to demonstrate the presence of direct projections from the main olfactory bulbs to GnRH neurons but none from the accessory olfactory system.

While much less research has been carried out in non-rodent mammals effects of olfactory cues from sexual partners appear to primarily involve air-born odors. Thus in the pig, rabbit and ferret it is the main and not the accessory olfactory system that seems to be involved in the processing of partner odor (Hudson and Distel, 1986; Dorries et al., 1997; Kelliher et al., 1999). The predominant role of the main olfactory system in processing olfactory cues from rams has been confirmed by the effects of lesions or inactivation. Destruction of the olfactory epithelium by intranasal administration of zinc sulfate or inactivation of the cortical nucleus of the amygdala by local administration of lidocaine completely blocked the short-term LH response to ram odor (Gelez and Fabre-Nys, 2004; Gelez et al., 2004b). By contrast electro-cauterization of the vomeronasal organ, sectioning of the vomeronasal nerve or inactivation of the medial nucleus of the amygdala had no inhibitory effects, again suggesting that the accessory olfactory system is not necessary for the effect of male cues (Cohen-Tannoudji et al., 1989; Gelez et al., 2004b).

Another approach that has been used to reveal the neural circuitry involved in processing male odor cues has been by through quantifying the expression of Fos protein in neurons as a molecular marker of cerebral activation (Hoffman et al., 1993). Using this approach we have shown that in adult experienced ewes male cues activate both the main and the accessory olfactory systems, although effects are much stronger within the main system (Gelez and Fabre-Nys, 2006). These findings are illustrated in **Figure 3**. In the main olfactory bulb, the cortical nucleus of the amygdala and the hippocampal dentate gyrus activation is relatively selective since Fos expression was increased to a greater extent after exposure to ram fleece than after exposure to female fleece (**Figure 3A**). In the piriform and entorhinal cortices, that are relays of the main olfactory system (Keverne and Winans, 1981; Jansen et al., 1998), male and female odors induced higher Fos expression than the control situation, suggestive of a more general response to socio-sexual stimulation. In the accessory olfactory bulb on the other hand while Fos expression was higher after exposure to male odor than after the control situation it showed a similar response to female odor, indicating that the accessory olfactory system may respond more generally to social odors rather than in selective detection of those associated with males. These results are however challenged by a recent study which found Fos activation in the accessory but not the main olfactory system of St Croix ewes in response to extracts of hair from male goats that induce an increase in LH secretion (Ohara et al., 2014).

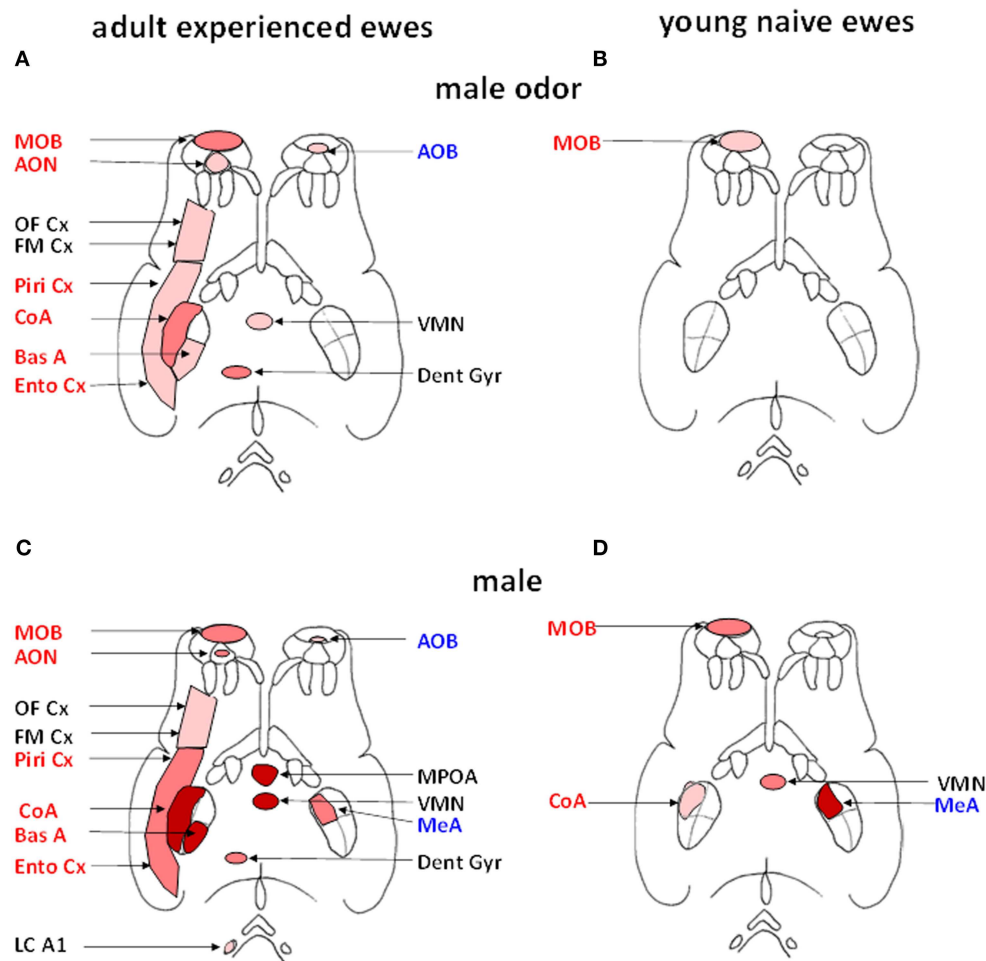


FIGURE 3 | Schematic representation of the brain regions activated (Fos immunoreactive, Fos IR) after exposure to male fleece (A, B) or to male odor (C, D) in adult ewes (A, C) or young naïve ewes (B, D).

Regions belonging to the main olfactory system are shown in red on the left side of each panel and those belonging to the accessory olfactory system in blue on right side (adapted from Jansen et al., 1998). Within each of the four diagrams, regions shown in red are those in which the density of Fos IR cells is significantly greater than in ewes exposed to ram fleece. Regions shown in dark pink are those in which the density of Fos IR cells in treated ewes is

significantly greater than in ewes exposed to the fleece of an unfamiliar ewe. Regions shown in pale pink are those in which the density of Fos IR cells is significantly higher than in ewes exposed to the test pen only. AOB, accessory olfactory bulb; AON, accessory olfactory nucleus; BasA, basal nucleus of the amygdala; CoA, cortical nucleus of the amygdala; Dent Gyr, dentate gyrus; Ento Cx, entorhinal cortex; FM Cx, frontomedial cortex; LC A1, locus coeruleus complex and A1 nucleus; MeA, medial nucleus of the amygdala; MPOA, medial preoptic area; OF Cx, orbitofrontal cortex; Piri Cx, piriform cortex; VMN, ventromedial nucleus of the hypothalamus.

However, the physiological relevance of these intriguing results remains to be established. Interestingly male odors also activate a number of other brain regions known to have more generalized roles in cognitive, emotional and reproductive functions including the basal amygdala, frontomedial cortex and ventromedial nucleus of the hypothalamus. Thus male odors may potentially influence a range of female behavioral responses as well as the gonadotropic axis.

Specific activation of the hippocampal dentate gyrus by male odor together with the olfactory bulb (Gelez and Fabre-Nys, 2006) is indicative of either formation and/or recall of a social recognition memory since both regions are known to play a key role in this respect (Brennan and Kendrick, 2006; Sanchez-Andrade and Kendrick, 2009). Contact with a ram has also been

reported to stimulate greater cellular proliferation in ewes compared to when they are left alone (Hawken et al., 2009b). The main source of axons to the dentate gyrus is the perforant path that arises from the entorhinal cortex and intrinsic connections with the rest of the hippocampus (Treves et al., 2008). Thus differential processing of odor cues from individual rams by olfactory and hippocampal regions may in turn impact on the ultimate effects that their individual cues have, or simply whether they are familiar or not, on the subsequent activation of GnRH neurons.

We have also found that experience modifies the extent of activation within the olfactory system in response to male odor cues. Thus, exposure to rams has very limited effects in young and sexually naïve ewes by comparison with experienced ones

(Chanvallon and Fabre-Nys, 2009). In these inexperienced ewes the only region more strongly activated by male than by female odors is the first relay of olfactory inputs, the main olfactory bulb (**Figure 3B**). Thus in the absence of any previous experience with rams, the link between their specific olfactory cues and limbic and hypothalamic regions mediating effects on GnRH neurons and reproduction are absent or weak, although the animals are capable of discriminating their odors.

As discussed above, actual contact with rams is generally more potent than simple exposure to their fleece in inducing the “ram-effect.” In support of this it has been found that such direct contact with a ram does indeed induce greater activation in many brain regions in contrast to exposure to male odor alone, or female odor, or control situations. Regions showing such enhanced activation include the preoptic area (MPOA), the ventromedial nucleus (VMN), the medial (MeA), cortical (CoA) and basal nuclei of the amygdala (**Figure 3C**). Importantly, destruction of the olfactory epithelium or its inactivation by lidocaine fail to prevent an increase in LH pulsatility when adult ewes are exposed to direct contact with a ram (Gelez et al., 2004b). Thus it would appear that male cues involving other sensory modalities must be contributing in some way. However, to date we have found no evidence for activation in the visual association cortex of anestrus animals in response to visual cues from rams (Gelez and Fabre-Nys, 2006) in agreement with the limited effect of exposure to images of rams on LH secretion in anestrus ewes (Hawken et al., 2009a). This is in contrast to the situation in estrus when association visual cortex is activated (Ohkura et al., 1997) and visual cues from ram faces can induce neurochemical changes in the MBH (Fabre-Nys et al., 1997). Similarly we have found no evidence for activation in auditory brain regions.

The enhanced neural activation pattern seen in response to actual male cues as opposed to odor cues alone also shows an impact of experience. Thus in young and sexually naïve ewes Fos expression changes were only detected in the main olfactory bulb, the cortical and medial nuclei of the amygdala and the ventromedial nucleus of the hypothalamus (**Figure 3D**) (Chanvallon and Fabre-Nys, 2009). This is more extensive than seen in response to male fleece, where only activation in the olfactory bulb was found. Indeed, the most notable difference between responses to the ram, as opposed to only its fleece, in naïve animals was in the strength of olfactory bulb activation. An important role of experience may therefore be to enhance the response of the olfactory bulb to male cues resulting in a more extensive pattern of activation in cortical, limbic and hypothalamic regions. This experience-dependent increased activation in the olfactory bulb may reflect learning of odor (Shea et al., 2008; Sanchez-Andrade and Kendrick, 2009; Tong et al., 2014) and other characteristics of rams and strengthened interactions with other downstream projection regions.

An increasing amount of research has focussed on hypothalamic and preoptic region circuitry involved in translating the information from male cues conveyed by projections from olfactory and limbic regions. Direct contact with a ram or exposure to ram odor increases the percentage of GnRH cells expressing Fos in the POA and OVLT of adult experienced ewes (Gelez and Fabre-Nys, 2006) but not in young naïve ones (Chanvallon

and Fabre-Nys, 2009). This is clearly consistent with the different responses to the “ram effect” observed in experienced and sexually naïve ewes. Although increased Fos expression also occurs in the hypothalamic ventromedial nucleus after exposure to either the ram or its odor, inactivation of this structure has no effect on the LH response to either ram odor or the ram itself. This suggests that this region, which is also activated in naïve ewes, may play a more general function in responding to cues from the ram, possibly related to increased attention (Gelez and Fabre-Nys, 2004).

An important question is therefore whether information from male odor and other cues is relayed directly to GnRH neurons, or by a more indirect route? In mice there is a direct connection between the main olfactory bulb and GnRH neurons (Yoon et al., 2005), so it is possible that part of the activation of GnRH neurons observed in ewes is due to such a direct connection, although this has yet to be established. On the other hand, in recent years many studies have shown that kisspeptin neurons have a major role in the control of GnRH secretion, and provide an important link between the GnRH neurons and sex steroids. Kisspeptin neurons may also mediate the effects of many other factors which influence the HPG axis (see review by Pinilla et al., 2012).

So are kisspeptin neurons the target for olfactory and limbic system projections involved in processing male cues important for mediating the “ram effect?” In sheep, as in other species, there are two populations of kisspeptin neurons, one in the preoptic area and another in the arcuate nucleus (Franceschini et al., 2006; Mikkelsen and Simonneaux, 2009). It was first considered that the two populations had different physiological roles (see review by Lehman et al., 2010); the preoptic area population of kisspeptin neurons in the LH surge and the arcuate population in the control of the pulsatile secretion of GnRH. However, a recent study has shown that in sheep the arcuate population of kisspeptin also has a role the LH surge (Merkley et al., 2012).

A potential direct functional role for kisspeptin neurons in mediating the “ram effect” is indicated by the finding that intracerebroventricular (ICV) administration of a kisspeptin antagonist (P271) 1 h before the introduction of rams prevents the increase in pulsatile LH secretion in response to them (De Bond et al., 2013). Furthermore, an increased proportion of kisspeptin neurons express Fos after ewes are exposed to a ram as opposed to an unfamiliar ewe (Ghenim et al., 2012), or not exposed to any other sheep (De Bond et al., 2013). Both populations of kisspeptin neurons seem to be involved and exposure to a ram for 2 h (Ghenim et al., 2012) or 3 h (De Bond et al., 2013) results in a 10 fold increase in the proportion of kisspeptin neurons expressing Fos in the arcuate nucleus. We also found an increase in the preoptic area although it was less marked (38%) than in the arcuate population, with 71% of the kisspeptin neurons expressing Fos-IR after 2 h of contact with a ram (Ghenim et al., 2012). De Bond et al. (2013) on the other hand failed to detect any preoptic changes. The recruitment of kisspeptin neurons in both locations is rapid and does not change significantly when the duration of exposure to rams is extended to 12 h. This suggests that the kisspeptin neurons become involved very soon after the first contact between the sexual partners. The involvement of kisspeptin

neurons may also last for some time because Fos protein normally disappears a few hours after transient stimulation (Hoffman et al., 1993), whereas in our experiment 38% of kisspeptin neurons in the arcuate population and 67% in the preoptic area population still contained Fos-IR in ewes exposed to the ram for 12 h (Ghenim et al., 2012).

Thus while the precise neural circuitry involved in mediating the ram effect requires further confirmation, a working hypothesis at this stage is that odor cues from males are processed primarily by a core circuit involving the main olfactory bulb and cortical amygdala and influence both preoptic and hypothalamic kisspeptin neurons which in turn then activate GnRH neurons (see Kendrick, 2014). Kisspeptin and/or GnRH neurons may also be more indirectly influenced by other cortical, limbic and hypothalamic regions responding to olfactory or other cues produced by the male.

Classical Neurotransmitters Involved

The classical neurotransmitter systems via which socio-sexual stimulation modulates GnRH neurons are only poorly understood. The best documented is the noradrenergic system which is involved in male-stimulated ovulation in rabbits and ferrets (Wersinger and Baum, 1997; Yang et al., 1996, 1997). In rabbits for example there is a parallel increase in noradrenalin (NA) and GnRH in the MBH within 10 min of mating (Kaynard et al., 1990). Furthermore, ICV infusions of the $\alpha 1$ adrenergic receptor antagonist prazosin, or administering it directly into the arcuate median eminence, either suppressed or reduced the post-coital GnRH and LH surge surges (Yang et al., 1998).

In sheep, when anestrus ewes are exposed to rams the rapid increase in LH is paralleled by a nearly 10-fold increase in NA concentrations in the posterior part of the medial preoptic area (MPOA, Figure 4), suggesting that evoked NA release in this region may be influencing GnRH neurons to promote subsequent LH release (Fabre-Nys et al., 2005, Supplementary Material). However, interestingly when estrus ewes are exposed to a ram, or just to a picture of its face, increased NA release is observed in the MBH rather than the MPOA (Fabre-Nys et al., 1994, 1997), although the onset of increased concentrations together with their amplitude and duration are very similar in the two contexts. These findings overlap to some extent with those in the rabbit where post-coital GnRH surges in females are also associated with increased NA release in the MBH but not in the anterior hypothalamus (Kaynard et al., 1990). Thus there may be some subtle differences in the ways that male cues alter LH release when animals are in anestrus as opposed to estrus, possibly associated with differing reproductive hormone profiles.

Direct manipulation of the noradrenergic system in the posterior MPOA during the ram effect using localized retrodialysis infusions has further confirmed its role in the response to male cues (Fabre-Nys and Scaramuzzi, 2014, Supplementary Material). Infusion of NA into the posterior preoptic area increased the proportion of ewes responding to a handful of ram fleece that by itself had a sub-threshold stimulating effect (10/11 vs. 5/10 animals). The frequency of LH pulses was also increased following NA infusions whereas the ram odor alone had no significant effects in

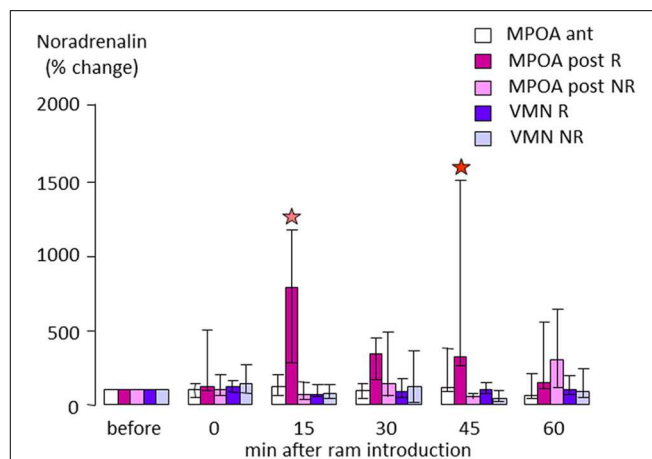


FIGURE 4 | Changes in extracellular noradrenaline concentration in the anterior (ant) or posterior part (post) of the medial preoptic area (MPOA) or in the ventromedial nucleus (VMN) of anestrus ewes that either responded (R) or did not respond by an increase in LH pulsatile secretion when exposed to a ram (NR). Samples were collected by microdialysis every 15 min and measured by electrochemical detection as in Fabre-Nys et al. (1994). Data are presented as percentage changes compared to the mean concentration during the three samples before male introduction. Data were compared using Friedman analysis of variance followed (when $p < 0.05$) by Dunn's multiple comparisons test a red star indicates that the post-treatment mean is different from the pretreatment mean ($p < 0.05$) and a pink star that it tended to be different ($p = 0.054$).

control ewes. Infusion of the $\alpha 1$ antagonist Prazosin in the posterior MPOA did not affect the proportion of ewes responding to the “ram effect” (7/11 vs. 8/11), and LH pulse frequency was increased both in control and Prazosin-treated ewes. However, the frequency and the amplitude of LH pulses following the “ram effect” were significantly reduced following Prazosin compared to the controls. These findings are somewhat similar to those in the rabbit where Prazosin infusions into the arcuate median eminence region only attenuated post-coital GnRH and LH surges, whereas ICV infusions completely suppressed them (Yang et al., 1998). Thus it is possible that NA is acting at multiple sites via the $\alpha 1$ receptor to influence LH secretion and ovulation both in the context of male-induced ovulation in female rabbits, and the ram effect in anestrus ewes.

In sheep, as in other species, noradrenergic neurons have their cell bodies within the pons and medulla of the brainstem (Tillet and Thibault, 1989) and some of them project to the MPOA and the MBH (Tillet et al., 1993). The proportion of tyrosine hydroxylase (TH), the rate limiting enzyme for the synthesis of NA, immunoreactive cells that were also immunoreactive for Fos in the A1 and in the locus coeruleus (LC) complex (A6–A7) is higher in ewes exposed to a ram than in controls (Fabre-Nys and Scaramuzzi, 2014, Supplementary Material). This suggests that noradrenergic afferents are involved in the response to male cues in anestrus ewes, in a similar way to that observed in rabbits and ferrets after mating (Kaynard et al., 1990; Wersinger and Baum, 1997; Yang et al., 1997, 1998). The noradrenergic projection from the LC to the olfactory bulb has been shown to be important in olfactory recognition memory (Brennan and Kendrick, 2006;

Sanchez-Andrade and Kendrick, 2009) and may also play a role in increasing sensitivity of the olfactory bulb to weak odors (Jiang et al., 1996). Furthermore, in the context of maternal ewes recognizing their lambs there is an experience dependent enhancement of olfactory bulb NA release (Lévy et al., 1993). Locus coeruleus stimulation has also been shown to augment MPOA stimulated GnRH release in rodents (Gitler and Barraclough, 1987). Thus in the context of odor stimuli from rams the LC noradrenergic projections to the olfactory bulb and to the MPOA may play a key role both in experience dependent perception and recognition of these odors at the level of the olfactory bulb and in facilitating GnRH release at the level of the MPOA. Importantly, since the LC is also associated more generally with relaying arousal and autonomic changes to widespread regions of the forebrain (Sara and Bouret, 2012), it is in a position to signal more general responses to the actual presence of a ram beyond those relating to specific odor molecules from its wool alone. Thus the LC projections may play a role in mediating more general influences of male cues on both reproductive and odor processing functions.

An important unanswered question at this stage is clearly how altered noradrenergic signaling modulates the activity of GnRH neurons (Herbison, 1997; Goodman et al., 2002; Clarke et al., 2006; Szawka et al., 2013). If inputs to the hypothalamic and preoptic area conveying information about ram cues involve noradrenergic signaling then one might expect from our above discussion of the potential neural circuitry mediating the ram effect that there would be some interaction with kisspeptin neurons. While this has not been shown directly a recent report on *Kiss1* knockout rats has shown that they fail to show LH release in response to either noradrenergic or glutamatergic stimulation (Uenoyama et al., 2015). Thus noradrenergic involvement in the ram effect might indeed be partly via an interaction with kisspeptin signaling, although further studies are clearly required to establish this.

Potential Scenarios of Events during the Ram Effect

The results obtained so far allow us to suggest a model of the sequence of events when anestrus ewes are exposed to a sexually active rams (Figure 5).

Step 1: The ram using principally odor cues and socio-sexual behavior patterns activates the main olfactory bulb and to a lesser extent the accessory olfactory system, of the courted ewe. This activation is transmitted along relays in the amygdala and the associated cortices (piriform, entorhinal cortices) and also to several cortical areas that have broader “cognitive” functions in terms of associative learning (orbitofrontal and frontomedian cortices) and to the noradrenergic system. Activation of these structures depends on the ewe’s previous experience with rams and the olfactory bulb and dentate gyrus probably have an important role in learning or remembering both male cues in general and also those of specific individuals.

Step 2: The brain regions that have been activated by ram cues in turn stimulate the GnRH network at least partly via activation of kisspeptin neurons. Factors such as stress or nutrition

might also interact at this level to modulate the activation of the GnRH network.

Step 3: Activated GnRH neurons secrete GnRH that induces short-term pulsatile secretion of LH. Most ewes will show this response but the frequency and amplitudes of the pulses can be modulated by several environmental factors; stress, nutrition, photoperiod and socio-sexual experience, and also by variation in the sensitivity of the gonadotrophic axis to estradiol feedback. The increased pulsatile secretion of LH stimulates ovarian follicles to secrete estradiol. In some ewes there are no follicles mature enough to respond to LH and so they will not secrete sufficient estradiol to induce a LH surge. In these ewes the action of the “ram effect” will be arrested at this stage.

Step 3b: In some ewes exposure to a ram immediately induces a LH surge (“precocious” LH surges) without the need for a period of increased pulsatile secretion of LH. We suggest that NA is involved in the induction of “precocious” LH surges. Ewes with higher activity of the noradrenergic system or a higher sensitivity to noradrenergic inputs may be more likely to have “precocious” LH surges.

Step 4: Estradiol secreted by responsive follicles stimulates preovulatory secretion of GnRH and LH surges as occurs in cyclic animals, leading to a LH surge and ovulation. Differences in sensitivity to estradiol result in ewes displaying different latencies in terms of the onset of their LH surge.

What the Ram Effect Could Tell Us About the HPG Axis

In many species, social interactions are major contributors to the adaptation of reproduction to a changing environment. This is the case for sheep and many other ungulate species in which the introduction of a male into a group of females in a reproductively quiescent state will reinstate cyclicity. These socio-sexual effects act by the modulation of GnRH secretion. In this last section we will propose a few questions that could be addressed either directly or indirectly using the “ram effect” as the experimental model in a new and fruitful way.

Pulsatile Secretion of GnRH

A Japanese group has already shown in very elegant studies in goats that the effects of male stimuli in goats can help unravel the details of pulsatile GnRH secretion (Hamada et al., 1996; Ichimaru et al., 2008). Their techniques have been developed using a miniature Shiba goat. However, the sheep is a species in which it is possible to study GnRH secretion directly by sampling hypothalamo-pituitary portal blood (Caraty and Locatelli, 1988). Because of the over-riding significance of ovarian steroids in the control of pulsatile GnRH secretion most studies have focused on this. The “ram effect” modulates pulsatile LH secretion and so probably also the pulsatile secretion of GnRH, although this has never been tested.

The “ram effect” is most potent when ewes are anestrus and more sensitive to the influence of negative estradiol feedback (Goodman et al., 1981, 2002). Furthermore, rams can also influence reproductive neuro-endocrine function in ewes during the

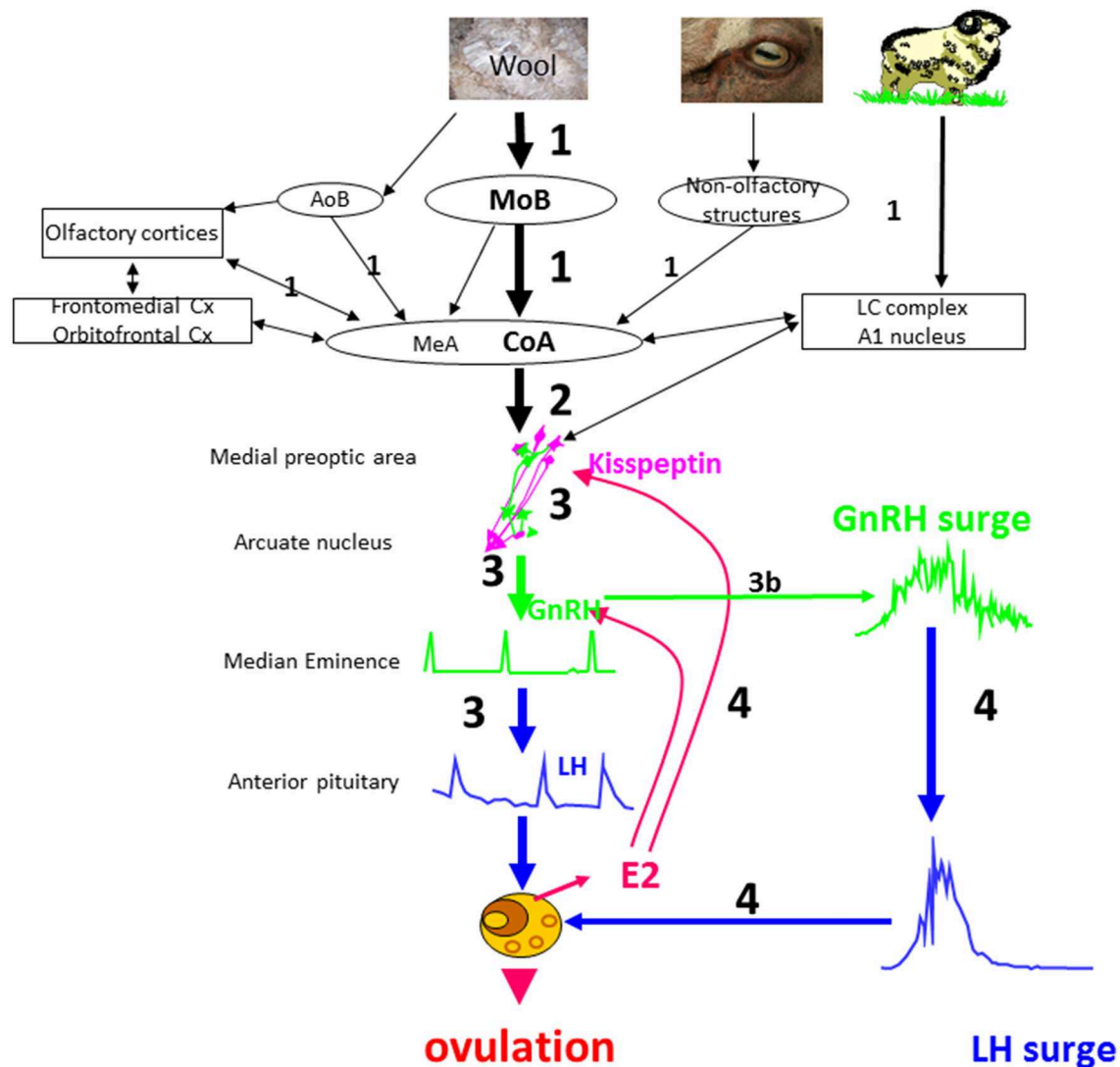


FIGURE 5 | Schematic illustration of a proposed model for the sequence of neuroendocrine events in ewes following the “ram effect”. The numbers indicate the chronology of events and their size the relative importance of a specific step. In a small proportion of ewes the step

3 leads directly to a LH surge (step 3b) without the need for estradiol positive feedback. MOB, main olfactory bulb; AOB, accessory olfactory bulb; CoA, corticomedial nucleus of the amygdala; Cx, cortex; MeA, medial nucleus of the amygdala; LC, locus coeruleus complex.

luteal phase when some progesterone is present, although this steroid is known to have a very strong negative feedback effect on GnRH pulsatile secretion (Goodman, 1996; Goodman et al., 2011). So clearly exposure to male cues can in some situations override the negative feedback effect of steroids. The mechanisms involved are currently unknown but it would be interesting to test if the control of GnRH secretion by ram cues is through a different neural network to that involved in negative feedback. Experiments in mice have identified a direct link between male odor and GnRH neurons (Boehm et al., 2005; Yoon et al., 2005), although demonstrating such a direct link in ewes may be difficult. Furthermore, considering the number of brain regions that are activated when ewes are exposed to rams it is very likely that many more of them could have direct or indirect links with the

medial preoptic area and the GnRH neuronal network than are known currently. Studies on the “ram effect” could help discover these unknown connections.

The LH Surge

The induction of a LH surge by exposure of anestrus ewes to a ram is nearly always due to an increase in estradiol secretion. The mechanism involved is most likely the same as the one that induces the preovulatory LH surge in spontaneous ovulators. However, in a few ewes the LH surge is induced immediately after rams are introduced suggesting a different mechanism and one closer to that in induced ovulators (Fabre-Nys et al., 2013). Several authors have suggested that the dualistic theory of spontaneous or induced ovulation is an over simplification and that

both systems could coexist in most females. To date very few attempts have been made to test this hypothesis and to unravel the two types of neural circuitry that would be required to explain the duality of induced and spontaneous ovulation. The identification of factors associated with “precocious” LH surges could help identify these neural circuits.

Role of Noradrenalin

Noradrenalin is clearly involved in the male induced LH surge in induced ovulators but has a more “permissive” role in the control of GnRH secretion in spontaneous ovulators. Direct connections between GnRH neurons and NA terminals have been described in many species and since GnRH neurons possess noradrenergic receptors the action of NA on them could be direct. However NA is released in a variety of circumstances and mediates changes both in attention and general arousal. The mechanisms of these effects are not clear and the hypothesis that some of these actions could take place through the kisspeptin neurons has not been tested, although this would be an interesting possibility.

Study of the Impact of Environmental Factors

A number of environmental factors (e.g., nutrition or stress) can modulate the response to the “ram effect” and in many cases the target of this modulation is the pulsatile secretion of GnRH. Indeed, some factors are known to have direct modulatory effects on the GnRH network (Chand and Lovejoy, 2011;

Roa and Tena-Sempere, 2014) and the mechanisms involved are starting to be understood, although, exactly how these factors interact with the effect of the male is not known. Understanding these interactions would help provide a more thorough understanding of the impact of environmental factors on reproduction.

Effect of Experience

Young ewes that are sexually naive have weaker physiological responses to the ram or to ram odor and the socio-sexual cues from the ram are less able to activate brain regions than in sexually experienced ewes. The physiological response of sexually naive ewes to the ram is enhanced if the ewes have had previous contact with sexually mature rams so that they could “learn” their characteristics. In a physiological sense, exactly what is meant by “learned” and how can sexual experience modulate the GnRH network? To our knowledge, this has never been studied, but the “ram effect” would certainly be a useful and potentially fruitful model to address this question.

Supplementary Material

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fnins.2015.00111/abstract>

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Reporter cell lines to evaluate the selectivity of chemicals for human and zebrafish estrogen and peroxysome proliferator activated γ receptors

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Zebrafish is increasingly used as an animal model to study the effects of environmental nuclear receptors (NRs) ligands. As most of these compounds have only been tested on human NRs, it is necessary to measure their effects on zebrafish NRs. Estrogen receptors (ER) α and β and peroxysome proliferator activated receptor (PPAR) γ are main targets of environmental disrupting compounds (EDCs). In humans there are two distinct nuclear ERs (hER α and hER β), whereas the zebrafish genome encodes three ERs, zfER α , zfER β 1, and zfER β 2. Only one isoform of PPAR γ is expressed in both humans and zebrafish. In this review, we described reporter cell lines that we established to study the interaction of EDCs with human and zebrafish ERs and PPAR γ . Using these cell lines, we observed that zfERs are thermo-sensitive while zfPPAR γ is not. We also showed significant differences in the ability of environmental and synthetic ligands to modulate activation of zfERs and zfPPAR γ in comparison to hERs and hPPAR γ . Some environmental estrogens (bisphenol A, mycoestrogens) which are hER panagonists displayed greater potency for zfER α as compared to zfER β s. hER β selective agonists (8 β VE2, DPN, phytoestrogens) also displayed zfER α selectivity. Among hER α selective synthetic agonists, 16 α -LE2 was the most zfER α selective compound. Almost all zfPPAR γ environmental ligands (halogenated bisphenol A derivatives, phthalates, perfluorinated compounds) displayed similar affinity for human and zebrafish PPAR γ while pharmaceutical hPPAR γ agonists like thiazolidones are not recognized by zfPPAR γ . Altogether, our studies show that all hERs and hPPAR γ ligands do not control in a similar manner the transcriptional activity of zfERs and zfPPAR γ and point out that care has to be taken in transposing the results obtained using the zebrafish as a model for human physiopathology.

Keywords: estrogen receptor, peroxysome proliferator activated receptor γ , environmental disrupting compounds, reporter cell lines, human, zebrafish

Introduction

Human nuclear hormone receptors (NHRs) are a family of 48 transcription factors, many of which have been shown to be activated by ligands. NHRs regulate cognate gene networks involved in key physiological functions such as cell growth and differentiation, development, homeostasis, or metabolism (Gronemeyer et al., 2004; Germain et al., 2006). Consequently, inappropriate exposure to environmental pollutants often leads to proliferative, reproductive, and metabolic diseases, including hormonal cancers, infertility, obesity or diabetes. NHRs are modular proteins composed of several domains, most notably an N-terminal domain, which harbors a ligand-independent activation function (AF-1), a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD) hosting a ligand-dependent transcriptional activation function (AF-2) (Gronemeyer et al., 2004). In the absence of the cognate ligand, some NHRs are located in the nucleus, bind to the DNA response elements of their target genes, and recruit corepressors, while others are located in the cytoplasm in an inactive complex with chaperones.

Ligand binding induces major structural alterations of the receptor LBDs, leading to (1) destabilization of corepressor or chaperone interfaces, (2) exposure of nuclear localization signals to allow nuclear translocation and DNA binding of cytoplasmic receptors, and (3) recruitment of coactivators triggering gene transcription through chromatin remodeling and activation of the general transcription machinery. The crystal structures of many NHR LBDs have been determined, revealing a conserved core of 12 α -helices (H1–H12) and a short two-stranded antiparallel β -sheet (S1 and S2) arranged into a three-layered sandwich fold. This arrangement generates a mostly hydrophobic cavity in the lower half of the domain, which can accommodate the cognate ligand. In all hormone-bound LBD structures, the ligand-binding pocket (LBP) is sealed by helix H12. This conformation is specifically induced by the binding of hormones or synthetic agonists and is referred to as the “active conformation” because it allows the dissociation of corepressors and favors the recruitment of transcriptional coactivators (Bourguet et al., 2000; Renaud and Moras, 2000; Pike, 2006).

In contrast to agonist binding, interaction with antagonists prevents the correct positioning of helix H12, thus avoiding association with the LxxLL motifs of coactivators. The LBD also contributes to the modulation of the N-terminal AF-1 through interdomain crosstalk so that both AF-1 and AF-2 domains can recruit a range of coregulatory proteins and act individually or in a synergistic manner (Benecke et al., 2000; Bommer et al., 2002; Wilson, 2011).

Among nuclear receptors, ERs and PPAR γ are main targets of numerous synthetic substances released into the environment by human activities. These substances can act as endocrine-disrupting chemicals (EDCs) causing reproductive, developmental, metabolic, or neurological diseases as well as hormone-related cancers (Diamanti-Kandarakis et al., 2009). Many EDCs are man-made compounds, for example bisphenols, phthalates, parabens, dioxins, pesticides, alkylphenols,

organotins, polychlorinated biphenyls, or perfluoroalkyl compounds. Some natural EDCs can also be found in plants and fungi. Standard methods to study interaction of EDCs with these nuclear receptors use stable cell reporter gene assays based on human ERs and PPAR γ activation (Balaguer et al., 1999; Legler et al., 1999; Seimandi et al., 2005; Riu et al., 2011a). To address whether chemicals exert an effect at the organismal level, ER activity assays have been developed for zebrafish. In these animals, GFP reporter constructs are designed to act in certain tissues exclusively (such as liver or brain) (Kurauchi et al., 2005; Brion et al., 2012) or in all tissues of embryos and larvae (Gorelick and Halpern, 2011; Lee et al., 2012). Zebrafish has also been used as an *in vivo* model to study the effect of environmental compounds on PPAR γ (Riu et al., 2014). Zebrafish stores neutral lipid triglycerides in visceral, intramuscular, and subcutaneous adipocyte depots (Tingaud-Sequeira et al., 2012). Studies of the zebrafish embryo, which is optically transparent thus facilitating the labeling and detection of lipid depots using lipid staining (Minchin and Rawls, 2011), have shown that white adipose tissue appearance is correlated with size rather than the age of the fish. By using zebrafish as a PPAR γ ligand screening model, we have showed that halogenated-BPA analogs are potent inducers of lipid accumulation *in vivo* through PPAR γ signaling (Riu et al., 2014).

In order to evaluate the effects of environmental and pharmaceutical compounds on the transcriptional activity of zfERs and zfPPAR γ and to compare the data with their activity on hERs and hPPAR γ , we established human and zebrafish ERs and PPAR γ reporter cell lines in the same cellular context (Balaguer et al., 1999; Seimandi et al., 2005; Pinto et al., 2014; Riu et al., 2014). In HeLa cells stably expressing an ERE-driven luciferase reporter (HELN cells), we expressed the full-length hER α , hER β , zfER α , zfER β 1, and zfER β 2, respectively. Similarly, in HeLa cells stably expressing a GAL4RE-driven luciferase reporter (HG5LN cells), we expressed a fusion protein consisting of the hPPAR γ or zfPPAR γ ligand binding domain (LBD) and the DNA binding domain (DBD) of the yeast transcription factor GAL4 (GAL4-PPAR γ).

The resulting HELN-ERs and HG5LN PPAR γ cell lines were used to evaluate the effects of environmental compounds on gene transactivation by the five ERs and the two PPAR γ , and to compare these effects with results obtained on hER and PPAR γ orthologs. Since zebrafish is used as a model for studying the effects of environmental compounds *in vivo*, determining the transcriptional profiles of these compounds on the zfERs and zfPPAR γ is crucial to support the zebrafish model for ER- and PPAR γ -related studies and their extrapolation to the mammalian system.

Estrogen Receptors

Estrogen signaling is mainly mediated by the two estrogen receptors ER α (also called NR3A1) and ER β (also called NR3A2) (Jensen and Jordan, 2003; Dahlman-Wright et al., 2006) which play important roles in the growth and maintenance of various tissues such as the mammary gland, uterus, bones, or the cardiovascular system. Like most NRs, ERs bind as dimers

to DNA response elements in the promoter region of target genes and respond to the naturally occurring sex hormone 17 β -estradiol (E₂). Both hERs are widely distributed throughout the body, displaying distinct but overlapping expression patterns in a variety of tissues (Couse and Korach, 1999). hER α is primarily expressed in the uterus, liver, kidney, and heart, whereas hER β is preferentially expressed in the ovary, prostate, lung, gastrointestinal tract, bladder, and hematopoietic and central nervous systems (Kuiper et al., 1997). However, hER α and hER β are coexpressed in a number of tissues including the mammary gland, thyroid, adrenal, bones, and some regions of the brain. Although hER α and hER β share similar mechanisms of action, several differences in the transcriptional abilities of each receptor and distinct phenotypes between gene-null animals have been identified, suggesting that these receptors may regulate distinct cellular pathways (Curtis et al., 1996; Couse and Korach, 1999). Interestingly, when hERs are coexpressed, hER β exhibits an inhibitory action on ER α -mediated gene expression (Pettersson et al., 2000; Liu et al., 2002), so that hER β has been shown to antagonize several hER α -mediated effects including fat reduction and cell proliferation in breast, uterus, or prostate (Ogawa et al., 1998; Weihua et al., 2000; Lindberg et al., 2003). Furthermore, in addition to controlling the normal development and function of the reproductive system and other tissues, estrogens are key regulators of primary breast and prostatic cancer growth (Jensen and Jordan, 2003). Roughly 40% of human cancers require steroid hormones for their growth and the first-line therapy for treatment of hormone-dependent cancers is based on androgen and estrogen antagonists interacting with AR or ERs and shutting down the corresponding hormone-responsive pathway. Interestingly, ER β has been shown to antagonize ER α -mediated effects on cell proliferation in the breast, uterus, ovary, and prostate (Weihua et al., 2000; Lindberg et al., 2003; Ellem and Risbridger, 2009). In this regard, estrogens with selectivity for either ER subtypes may produce different biological outcomes, particularly on cancer cell proliferation. Given the widespread role of ERs in human physiology, it is not surprising that environmental compounds which bind to ERs, thus substituting for the natural hormone and deregulating the fine-tuned action of E₂, can lead to ER-related disorders including breast, endometrial, colorectal, or prostate cancers, as well as neurodegenerative, inflammatory, immune, cardiovascular, and metabolic diseases.

Small fish including zebrafish (*Danio rerio*) are increasingly being used as model species to study *in vivo* effects of EDCs (Segner, 2009; Vosges et al., 2010; Brion et al., 2012). In zebrafish, three zfER subtypes (zfER α , zfER β 1, and zfER β 2) are present (Menuet et al., 2002; Hawkins and Thomas, 2004). Zebrafish ER α (esr1) is orthologous to the human ER α , while ER β 1 (esr2b) and ER β 2 (esr2a) are orthologs of the human ER β (Bardet et al., 2002). The overall amino-acid sequence identity between the zfER subtypes and their corresponding human ER orthologs is approximately 50% (Menuet et al., 2002). ZfERs are differently expressed and regulated in reproductive tissue like gonads, liver, as well as in brain. In adult liver, E₂ induces zfER α expression while it has no effect on zfER β 2 and represses zfER β 1 expression (Menuet et al., 2002). Moreover, both zfER α and

zfER β 2 upregulate zfER α expression after E₂ exposure, whereas zfER β 1 has no effect on this expression (Menuet et al., 2004). These studies suggest that the different forms of zfERs have partially distinct and nonredundant functions. Hence, in the perspective of developing fish *in vitro* assays, it is essential to take into account all zfER subtypes in the assessment of chemical estrogenicity in zebrafish. Since these three zfERs are thought to mediate different biological effects, there is an increased interest in finding subtype-selective zfER ligands.

Estrogen Receptors Reporter Cell Lines

To understand and to evaluate impact of xenoestrogens on ER-signaling pathway, it is necessary to develop cell-based transcription assay systems that could reflect different cellular contexts and/or different model species. *In vitro* assays based on reporter gene driven by ERE have been proven to be useful and relevant screening tools to address the large number of chemicals yet needed to be tested for their estrogenic potential. We and other groups have developed stable reporter gene assays based on human ER α and ER β activation in different cell contexts and successfully used them to characterize estrogenic potency of chemicals (Balaguer et al., 1999; Legler et al., 1999; Wilson et al., 2004; Sotoca et al., 2008; Docquier et al., 2013). In order to take into account the species of origin of studied receptor in hazard assessment of estrogenic chemicals in fish, we have developed *in vitro* stable reporter gene assays derived from fish species (Molina-Molina et al., 2008; Cosnefroy et al., 2012; Pinto et al., 2014). Among them, HELN-zfER α , -zfER β 1, and -zfER β 2 (Pinto et al., 2014) reporter cell lines were established in a similar way than HELN-hER α and -hER β cell lines (Pinto et al., 2014). Briefly, HELN-ERs cell lines cells were obtained by transfection of HELN cells (HeLa cells stably transfected with the ERE- β Globin-Luc-SVNeo plasmid) (Balaguer et al., 1999) by the corresponding pSG5-puro plasmids (pSG5-hER α -puro, -hER β -puro -zfER α -puro, -zfER β 1-puro, and -zfER β 2-puro, respectively).

Selectivity of Chemicals for Human and Zebrafish Estrogen Receptors

Screening of endogenous, environmental and synthetic ligands in the HELN-zfER cell lines showed that known mammalian ER ligands are also able to induce transcriptional activity of zebrafish ER subtypes (Pinto et al., 2014). This screening allowed us to assess differences in the potency of the estrogenic compounds among the three zfER subtypes, and compare their selectivity toward hERs using a similar human cellular context. The HELN-zfERs cells were incubated at 28°C after addition of chemicals to the cells because it is a more physiologically relevant temperature for zebrafish, which increased the potency of estradiol approximately 10-fold compared to incubation at 37°C. Temperature sensitivity of fish ERs has already been reported using reporter gene assays (Matthews et al., 2002; Cosnefroy et al., 2009) and the reason seems to be thermodependence of estrogen binding (Tan et al., 1999; Matthews et al., 2002; Sumida et al., 2003).

We have shown that there are clear differences between the selectivity of various (anti)estrogens for zebrafish and human ER isoforms, establishing the fact that a direct translation of (anti)estrogenic effects (activities or potencies) from mammals to zebrafish is not possible. Although none of the tested compounds specifically activated either zebrafish or human ERs, transcriptional activities toward human and zebrafish ERs need to be studied.

Natural (E2) and pharmaceutical (EE2) estrogens display similar affinities for hERs and zfERs. Some environmental estrogens (α -zearalanol, bisphenol-A) with similar affinity for hERs preferentially activated zfER α rather than zfER β s. Other environmental estrogens (nonylphenol mixture, 4-tert-octylphenol) with similar affinity for hERs displayed slightly higher affinity for zfER α and zfER β 2 than for zfER β 1. Benzophenone 2 and phytoestrogens (genistein, liquiritigenin) which have higher affinity for hER β than for hER α also displayed slightly higher affinity for zfER α and zfER β 2 than for zfER β 1. Finally, hER β selective synthetic compounds (8 β -vE2, DPN) preferentially activated zfER α compared to zfER β s. On the contrary and similar to hERs, the synthetic compound 16 α -E2, which has 1000-fold more selectivity for hER α (Escande et al., 2006), also exhibited higher affinity for zfER α compared to the zfER β subtypes and is the most selective compound for zfER α nowadays (Figure 1; Table 1).

To gain structural insights into the zfER α selectivity of 16 α -LE2 in human and zebrafish ERs, we used the web-based server EDMon (Endocrine Disruptor Monitoring; <http://atome2.cbs.cnrs.fr/AT2B/SERVER/EDMon.html>) (Delfosse et al., 2012) to model zfERs in complex with this ligand. The structural

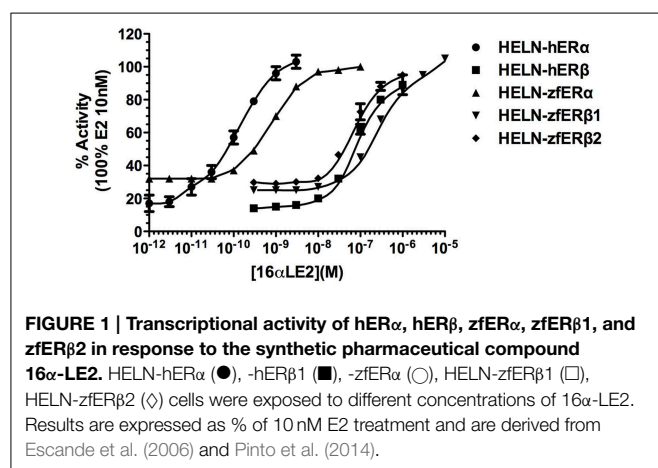
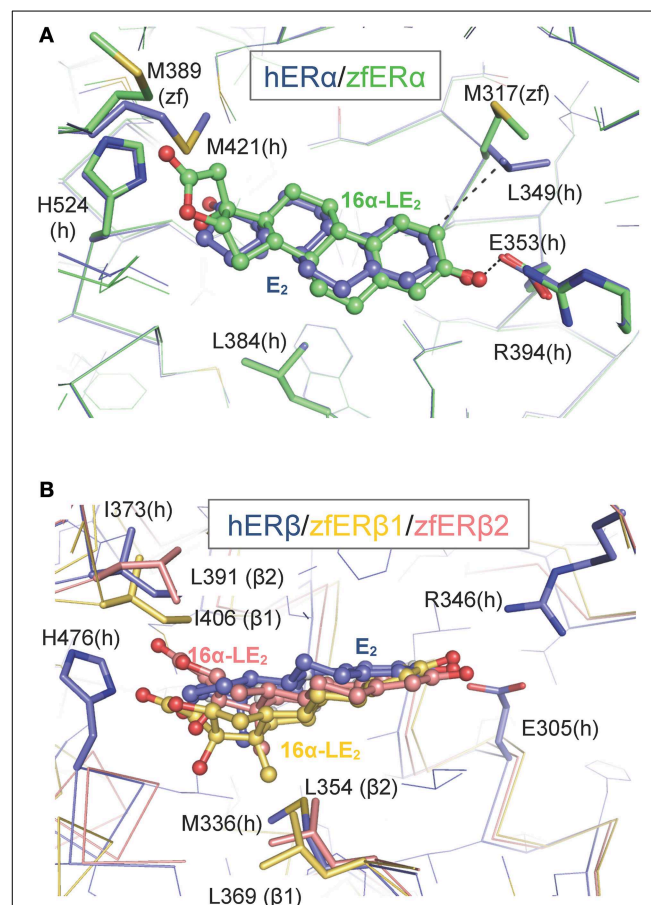


TABLE 1 | 16 α -LE2 ERs EC₅₀ and maximal activities.

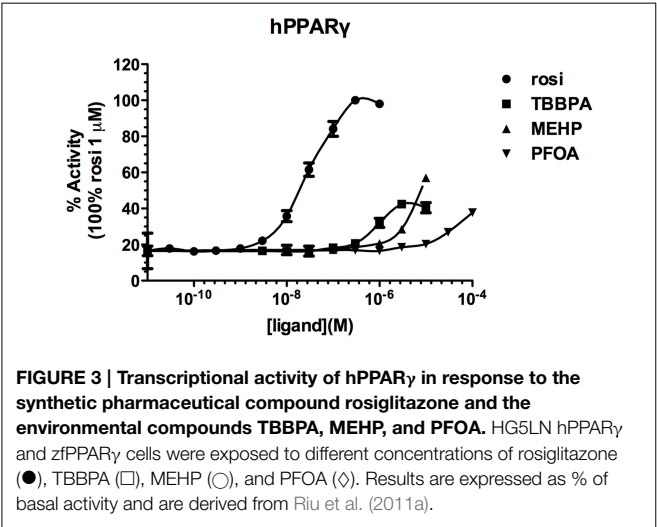
| NR | EC ₅₀ (nM) | Maximal activity (%) |
|----------------|-----------------------|----------------------|
| hER α | 0.093 \pm 0.025 | 100 |
| hER β | 92.9 \pm 1.4 | 100 |
| zfER α | 0.79 \pm 0.43 | 100 |
| zfER β 1 | 240 \pm 30.2 | 100 |
| zfER β 2 | 74.6 \pm 7.08 | 100 |

basis of the hER α and hER β selectivity toward certain ligands has been associated with two amino acid differences in their ligand-binding pockets. Indeed, L384 and M421 of hER α are replaced by M336 and I373 in hER β , respectively (Figure 2 and Manas et al., 2004). Superimposition of the 16 α -LE2-bound zfER α model on the crystal structure of hER α in complex with E2 (PDB code 3UUD) showed that the phenol ring of 16 α -LE2 occupies the same position as that of E2 and is engaged in a network of hydrogen bonds with E353 from helix 3 (H3) and R394 from H5 (Figure 2A). On the other side of the ligand-binding pocket (LBP), it appears that the hydrogen bond observed between the 17-hydroxyl group of E2 and H524 (H11) is conserved in 16 α -LE2. The difference



between the two complexes resides in the lactone ring of 16 α -LE2 which points toward M421 (H7) that must undergo a large conformational change to accommodate this additional group. In hER β , the linear M421 is replaced by the branched residue Ileu 373 characterized by a much smaller intrinsic flexibility (**Figure 2B**). As a consequence, I373 maintains the synthetic ligand in a position where it interacts unfavorably with M336 (H3). Therefore, 16 α -LE2 adopts different positions in hER α and hER β , the more constrained environment provided by the latter accounting for the weaker affinity of the ligand for this receptor subtype. The affinity values measured with the zebrafish receptors reflect the variations in the space constraints provided by the different combinations of residues in the three receptor subtypes. With H3 and H7 residues identical to those of the human receptor, zfER α interacts with 16 α -LE2 with the highest affinity. The slight difference in the binding affinity of 16 α -LE2 for hER α and zfER α relies most likely on the replacement of L349 (H3) by a methionine residue (M317) (**Figure 2A**) and a possible loss of a favorable interaction provided by the branched but not by the linear residue (**Figure 2A**). With a conserved isoleucine in H7 (I406) and a leucine residue in H3 (L369) (**Figure 2B**), zfER β 1 displays the most constrained LBP reflecting the weakest binding affinity for 16 α -LE2. This receptor combines two large residues with low (isoleucine) and medium (leucine) flexibilities. The replacement of I406 in H7 of zfER β 1 by a leucine residue (L391) (**Figure 2B**) in zfER β 2 provides a slight gain in LBP plasticity, in agreement with the slightly better affinity of 16 α -LE2 for the latter.

The inability of hER β -selective phytoestrogens (genistein and liquiritigenin) and pharmaceuticals (8bv-E2, DPN) to activate preferentially the zfER β isoforms is explained by the mutation of a critical amino acid involved in genistein binding in hER β . In all zfERs, the position homologous to hER β M336 is occupied, as in hER α , by a leucine residue (**Figure 2**) (Sassi-Messai et al., 2009). This amino acid change most likely accounts for the lack of obvious selectivity of the phytoestrogens toward the zfER β subtypes.



Peroxisome Proliferator γ

PPARs are involved in the regulation of glucose, lipid, and cholesterol metabolism in response to fatty acids and their derivatives, eicosanoids, and drugs used in the treatment of hyperlipidemia and diabetes. The human PPAR subfamily contains three members known as hPPAR α , hPPAR β , and hPPAR γ . Each hPPAR subtype shows a distinct tissue distribution and ligand preference. hPPAR γ is highly expressed in adipose tissue and is a central regulator of lipid storage and adipocyte gene expression and differentiation (Tontonoz et al., 1995) and is involved in various pathophysiological disorders, including metabolic disease, insulin resistance, and diabetes (Rosen and Spiegelman, 2001). hPPAR γ is the target for antidiabetic agents of the thiazolidinedione class, which includes troglitazone, pioglitazone, and rosiglitazone. The LBD of hPPAR γ is rather large and the diversity of ligands that can be accommodated within its pocket, mainly represented by lipid derivatives, may contribute to the large array of roles that have been assigned to hPPAR γ . Given the physiological role of hPPAR γ in adipose tissue development and maintenance, it has been proposed that disruption of regulation pathways under the control of hPPAR γ may be involved in the onset of diabetes and obesity (Swedenborg et al., 2009). Indeed, activation of this receptor by certain xenobiotic compounds has been shown to stimulate adipogenesis *in vitro* and *in vivo* through induction

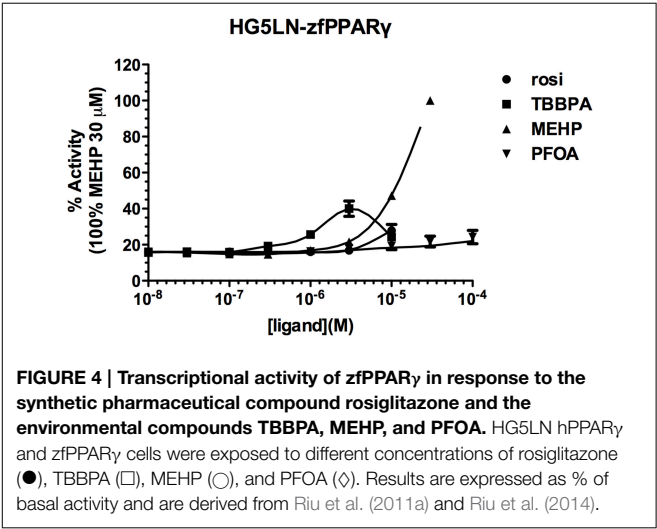


TABLE 2 | EC₅₀ and maximal activities of PPAR γ ligands.

| Ligand | hPPAR γ | zfPPAR γ |
|---------------|--|---|
| | EC ₅₀ (μM) (maximal activity %) | E ₅₀ (μM) (maximal activity %) |
| Rosiglitazone | 0.027 ± 0.003 (100) | ND (27.9) |
| TBBPA | 0.762 ± 0.136 (42.4) | 1.45 ± 0.33 (40) |
| MEHP | 1050 ± 73 (57) | 11.3 ± 1.29 (100) |
| PFOA | 380 ± 71 (37.8) | ND (24.2) |

ND, Not determined.

of the differentiation of preadipocytes of the fibroblastic lineage into mature adipocytes (Grun and Blumberg, 2009; le Maire et al., 2009; Janesick and Blumberg, 2011; Riu et al., 2011a). This has led to the “obesogen hypothesis,” according to which, in addition to disruption of the balance between caloric intake and expenditure characterizing modern life-style, the rapidly growing obesity epidemic could also implicate environmental risk factors including an increased exposure to chemicals that interfere with any aspects of metabolism (Grun and Blumberg, 2009; Janesick and Blumberg, 2011, 2012). Accordingly, compounds that have the potential to disrupt any metabolic signaling pathways and lead to increased fat accumulation and obesity are referred to as “obesogens” (Grun and Blumberg, 2006).

Like for ERs, zebrafish begin to be used as model species to study *in vivo* effects of EDCs on PPAR γ (Lyche et al., 2011; Riu et al., 2014). Similar to mammals, zebrafish store neutral lipid triglycerides in the visceral, intramuscular, and subcutaneous adipocyte depots. The first adipocytes, which can be observed from day 8 to 12, or at a minimal size of about 5 mm (Imrie and Sadler, 2010), appear in the pancreatic region, then in the viscera, and later on, in the subcutaneous and cranial regions (Flynn et al., 2009; Imrie and Sadler, 2010). Lipid staining can be detected before this stage; however, at this time point, the lipids

are not stored in adipocytes, but rather in the yolk, hepatocytes, blood vessels, skeletal myocytes, jaw chondrocytes, and neuronal tissue in the brain (Imrie and Sadler, 2010).

Ppar γ reporter cell lines

HG5LN-hPPAR γ and -zfPPAR γ reporter cell lines were established in a similar way (Seimandi et al., 2005; Riu et al., 2014). Briefly, HG5LN-PPAR γ cell line was obtained by transfection of HG5LN cells (HeLa cells stably transfected with the GALRE5- β Globin-Luc-SVNeo plasmid) (Seimandi et al., 2005) by the corresponding pSG5-puro plasmids [pSG5-GAL4(DBD)-hPPAR γ (LBD)-puro and -zfPPAR γ (LBD)-puro, respectively]. Interestingly, the thermodependence observed for zfERs is not shared by zfPPAR γ (Riu et al., 2014).

Selectivity of chemicals for human and zebrafish PPAR γ

Screening of environmental and pharmaceutical ligands in the HG5LN-zfPPAR γ cell lines showed that known hPPAR γ ligands are not always able to induce transcriptional activity of zebrafish

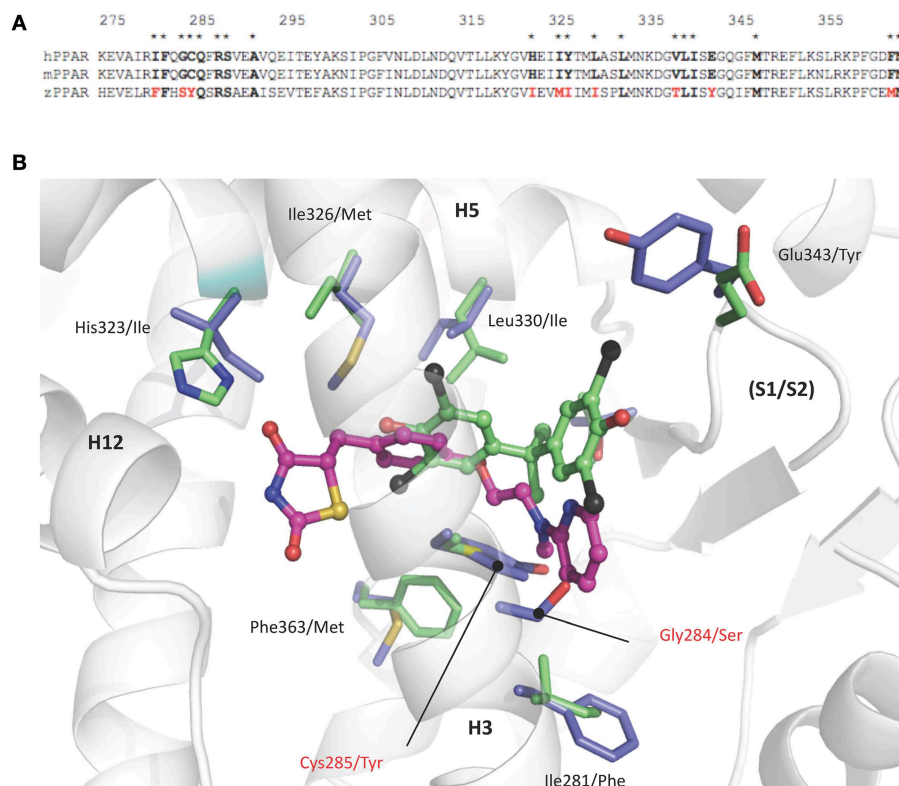


FIGURE 5 | (A) Sequence alignment of human, mouse, and zebrafish PPAR γ ligand binding pocket residues. Asterisks denote residues in contact with TBBPA and/or rosiglitazone (PDB code 2PRG). Interacting residues that differ between sequences are highlighted in red. **(B)** TBBPA (carbon atoms colored in green) and rosiglitazone (magenta, PDB code

2PRG) as they are positioned in the human PPAR γ . Residues that differ in the ligand binding pocket of human and zebrafish PPAR γ are displayed as green and blue sticks, respectively. The human PPAR γ Gly284 and Cys285 which are replaced by serine and tyrosine residues in zebrafish PPAR γ are indicated in red. This figure is derived from Riu et al. (2011a).

PPAR γ (Riu et al., 2011a). Pharmaceutical hPPAR γ ligands like thiazolidones (rosiglitazone, troglitazone) do not or very weakly bind to zfPPAR γ . On the contrary, environmental PPAR γ compounds including phthalates (MEHP), perfluorinated compounds (PFOA, PFOS) and halogenated derivatives of BPA (TBBPA, TCBPA) are common activators of hPPAR γ and zfPPAR γ (Figures 3, 4; Table 2). We also provide evidence that activation of ERs and PPAR γ depends on the halogenation degree of BPA analogs. The bulkier are brominated BPA analogs, the greater is their capability to activate PPAR γ and the weaker is their estrogenic potential (Riu et al., 2011b).

Comparison of human and zebrafish PPAR γ sequences reveals several residue differences which could explain the differential ligand specificity of the various species (Figure 5A). In particular, the replacement of human PPAR γ Gly284 and Cys285 by serine and tyrosine residues in zebrafish PPAR γ provides a rationale for the weak binding affinity of rosiglitazone for this receptor as compared to that observed for the human homolog (Figure 4B). In contrast, the different binding mode of halogenated compounds allows both hPPAR γ and zfPPAR γ to accommodate TBBPA and TCBPA (Figure 5B).

Structural and biophysical studies revealed that TBT binds to both hRXR and hPPAR γ through formation of a covalent bond between the tin atom and the sulfur atom of cysteine residues located in the LBP of both receptors (le Maire et al., 2009; Delfosse et al., 2014). In RXR, this cysteine (Cys432) is located in helix H11 and is conserved in several species. In contrast, the

cysteine residue of PPAR γ (Cys285) resides in H3 and is not conserved in several species including zebrafish.

Conclusion

We have shown above that there are clear differences between the activity of various EDCs for zebrafish and human ERs and PPARs, demonstrating that a direct translation of effects from mammals to zebrafish is not possible. The differences revealed in this study, in terms of transcriptional activities toward human and zebrafish ERs and PPARs, highlight the need to take into account the species of origin when assessing the potency of chemicals. This is particularly important with regard to EDCs screening for hazard assessment since at the present time established test guidelines are only based on human cell lines expressing human nuclear receptors.

To this end, such *in vitro* cell lines expressing zebrafish nuclear receptors can serve as useful screening tools to address nuclear receptor potency of chemicals for fish models. Hence, an initial screening should be followed up with an NR-subtype specific analysis using both human and zebrafish NRs to elucidate the full spectrum of NR-mediated EDCs effects.

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Melanocortin receptor accessory proteins in adrenal disease and obesity

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Melanocortin receptor accessory proteins (MRAPs) are regulators of the melanocortin receptor family. MRAP is an essential accessory factor for the functional expression of the MC2R/ACTH receptor. The importance of MRAP in adrenal gland physiology is demonstrated by the clinical condition familial glucocorticoid deficiency type 2. The role of its paralog melanocortin-2-receptor accessory protein 2 (MRAP2), which is predominantly expressed in the hypothalamus including the paraventricular nucleus, has recently been linked to mammalian obesity. Whole body deletion and targeted brain specific deletion of the *Mrap2* gene result in severe obesity in mice. Interestingly, *Mrap2* complete knockout (KO) mice have increased body weight without detectable changes to food intake or energy expenditure. Rare heterozygous variants of MRAP2 have been found in humans with severe, early-onset obesity. *In vitro* data have shown that *Mrap2* interaction with the melanocortin-4-receptor (Mc4r) affects receptor signaling. However, the mechanism by which *Mrap2* regulates body weight *in vivo* is not fully understood and differences between the phenotypes of *Mrap2* and *Mc4r* KO mice may point toward *Mc4r* independent mechanisms.

Keywords: melanocortin receptors, accessory proteins, adrenal function, obesity, knockout mouse model

The Melanocortin Receptor Family

Melanocortin receptors (MCRs) comprise a family of five, class A, G protein-coupled receptors designated MC1R-MC5R with diverse physiological roles. MCRs are found in chordates and are regarded as having evolved from a single ancestral receptor, possibly corresponding most closely to MC4R (Dores, 2013). Signaling by MCRs has primarily been observed as occurring through the stimulatory α unit Gs which activates adenylyl cyclase to produce cAMP, although other pathways have been implicated (Yang, 2011). Receptor properties such as biased agonism (MC4R) and constitutive activity (MC3R and MC4R) have also been reported (Nijenhuis et al., 2001; Breit et al., 2011).

The melanocortin agonist ligands for MCRs, adrenocorticotrophic hormone (ACTH) and the melanocyte stimulating hormones α MSH, β MSH, and γ MSH, are neuropeptides derived by enzymatic cleavage from proopiomelanocortin (Pritchard et al., 2002). Their relative potencies are set out together with the natural antagonists in **Table 1**. The natural peptide antagonists comprise agouti and agouti related protein (AgRP) (Cone, 2006). Agouti was first discovered as a high affinity MC1R antagonist, following studies on genetic determinants of yellow coat color and obese phenotype observed in agouti mouse strains (Lu et al., 1994). Agouti-related

TABLE 1 | Natural ligands of the melanocortin receptor family (Kiefer et al., 1997; MacNeil et al., 2002; Cone, 2006).

| Receptor | Relative potency of agonists | Antagonists | Main site of expression | Primary Function |
|----------|--|--------------|-------------------------|---|
| MC1R | α MSH = β MSH = ACTH > γ MSH | Agouti | Melanocytes | Pigmentation, inflammation |
| MC2R | ACTH only | | Adrenal cortex | Adrenal steroidogenesis |
| MC3R | α MSH = β MSH = ACTH \approx γ MSH | Agouti; AgRP | CNS, GI tract, Kidney | Energy homeostasis, inflammation, Entrainment to meal intake |
| MC4R | α MSH = β MSH = ACTH >> γ MSH | Agouti; AgRP | CNS | Energy homeostasis, thermogenesis, appetite regulation, erectile function |
| MC5R | α MSH > β MSH = ACTH >> γ MSH | | Exocrine cells | Exocrine function, regulation of sebaceous glands |

protein (AgRP) was later identified as a MC3R and MC4R antagonistic peptide almost identical in size and genomic structure to Agouti (Ollmann et al., 1997; Shutter et al., 1997). Since then it has been shown that both Agouti and AgRP can act as inverse agonists in cAMP assays inhibiting MC1R and MC4R constitutive activity respectively (Vage et al., 1997; Chai et al., 2003). However, such inverse agonists to the MC4R can act as agonists through intracellular ERK1/2 signaling (Breit et al., 2011; Mo and Tao, 2013). A number of additional molecules have been shown to alter MCR function in a variety of ways including β -defensin 3 (Beaumont et al., 2012; Swope et al., 2012), attractin (also known as mahogany), and mahogunin (He et al., 2001).

MC1R

Pigmentation and the control of the inflammatory functions of the immune system are dependent on MC1R. Stimulation of MC1R in skin and hair follicle cells by α MSH results in melanogenesis producing dark skin or hair in many species, including humans (Beaumont et al., 2011). Variants in MC1R in humans are associated with red hair, pale skin, and increased risk to skin cancer. This action of α MSH is antagonized *in vivo* by agouti (Lu et al., 1994). MC1R is present on human leukocytes, and on murine macrophages, and has been implicated in the anti-inflammatory properties of α MSH (Star et al., 1995; Catania et al., 2004).

MC2R

MC2R is unique as it is the only MCR that binds to a specific ligand ACTH. The receptor is predominantly expressed in the adrenal gland. In humans, the inability of ACTH to activate MC2R leads to failure of the adrenal gland to generate cortisol, resulting in the potentially lethal condition of familial glucocorticoid deficiency (FGD) (Clark et al., 1993). Mutation in MC2R leads to FGD type 1. The FGD phenotype was reproduced in the MC2R knockout mouse, although the mice also had evidence of mineralocorticoid deficiency (Chida et al., 2007). MC2R is also found in human and mouse bone where it is thought to be involved in osteoblast proliferation (Zhong et al., 2005). Interestingly, patients with FGD type 1 have tall stature (Elias et al., 2000). MC2R has also been detected in human subcutaneous fat (Smith et al., 2003) and the developing testis (O'Shaughnessy et al., 2007). In mouse adipose tissue, MC2R may be implicated in the release of non-esterified fatty acids from adipocytes (Moller et al., 2011).

MC3R

MC3R is primarily expressed in the central nervous system in the arcuate nucleus of the hypothalamus and limbic areas, where it affects food utilization/partitioning and food anticipatory behavior (Sutton et al., 2008, 2010; Begriche et al., 2011). Although loss-of-function mutations in MC3R have been identified in humans with obesity, there is still uncertainty if these variants are truly causative (Lee et al., 2007; Mencarelli et al., 2011). In mice, MC3R is essential for the maintenance of a circadian rhythm of activity related to feeding behavior (Begriche et al., 2012). Both central and peripheral MC3R are involved in energy utilization (Begriche et al., 2011). MC3R has also been implicated in the anti-inflammatory processes in murine macrophages (Getting et al., 2006; Leoni et al., 2008; Montero-Melendez et al., 2014).

MC4R

MC4R is thought to bind principally to α MSH in the paraventricular nucleus (PVN) of the hypothalamus (CNS), a region crucial in the control of food intake. Global homozygous deletion of MC4R in mice results in hyperphagia, increased fat and lean mass, increased body length, reduced activity, and reduced metabolic rate (Huszar et al., 1997; Balthasar et al., 2005). Inactivating mutations in MC4R are the single most common form of monogenic obesity in humans (Farooqi et al., 2003). Common variants near the MC4R locus are associated with adiposity, body weight, risk of obesity and insulin resistance at a population level (Chambers et al., 2008; Loos et al., 2008). The function of MC4R has also expanded over recent years and involvement in autonomic regulation of thermogenesis and glycaemia (Berglund et al., 2014), regulation of sympathetic and parasympathetic control of blood pressure (Sohn et al., 2013) and anhedonia (Lim et al., 2012) have all been described. A role in erectile function and sexual behavior have also been reported (van der Ploeg et al., 2002). Most recently, MC4R expression has been demonstrated in enteroendocrine L cells and regulates the release of peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) (Panaro et al., 2014).

MC5R

The need for MC5R activity in the secretory function of exocrine glands is well-known (Chen et al., 1997; van der Kraan et al., 1998). MC5R is widely expressed and mice deficient of Mc5r have impaired water repulsion and thermoregulation (Chen et al., 1997). There are some suggestions that MC5R expression in the zona glomerulosa of the adrenal gland may be involved in

melanocortin stimulated aldosterone secretion (Vinson et al., 1980; Griffon et al., 1994; van der Kraan et al., 1998), although this is not in keeping with the lack of an adrenal phenotype in Mc5r knockout mice (Chen et al., 1997). Stimulation of MC5R in 3T3-L1 adipocytes with α MSH has been shown to result in lipolysis, through cAMP production, and impairment of re-esterification of fatty acids, through the ERK1/2 pathway (Rodrigues et al., 2013).

The Melanocortin Receptor Accessory Proteins, MRAP, and MRAP2

Discovery of MRAP and MRAP2

The discovery of MRAP (sometimes referred to as MRAP1) in 2005 by Metherell et al. has provided insight into a novel aspect of MCR regulation, previously unknown (Metherell et al., 2005). By studying the clinical condition FGD it was noted that only approximately 25% of FGD cases were attributable to mutations of MC2R (Chan et al., 2009). Failure of MC2R to traffick to the cell surface in cells other than those derived from an adrenal lineage suggested the presence of an adrenal specific accessory protein (Noon et al., 2002). Using whole genome SNP array genotyping on informative families, mutations in the gene encoding a protein derived from open reading frame 61 of human chromosome 21 (C21orf61), corresponding to a murine adipocyte transmembrane protein (Xu et al., 2002), was identified to cause FGD type 2 (Metherell et al., 2005). This gene was subsequently named melanocortin-2-receptor accessory protein (MRAP). The human MRAP gene contains six exons, exons 5 and 6 are alternatively spliced to give rise to two MRAP isoforms, MRAP α (exon1–5), and MRAP β (exon 1–4 and 6). Exons 1 and 2 are not translated in the human isoforms whilst rodents do not have the corresponding exons 1 and 2 and produce only one form of the protein (Webb and Clark, 2010). The translated small single pass transmembrane domain protein differs from other known GPCR accessory proteins (Webb and Clark, 2010) and has been shown to adopt a unique anti-parallel homodimer conformation at the cell surface (Sebag and Hinkle, 2007; Cooray et al., 2008). Both isoforms are present with MC2R in human adrenal tissue (Metherell et al., 2005). The presence of either MRAP α or MRAP β is essential for MC2R cell surface expression and signaling (Metherell et al., 2005; Roy et al., 2007; Cooray et al., 2008; Hinkle and Sebag, 2009), but the response of MC2R to ACTH may differ between the two isoforms, with MRAP α increasing potency but MRAP β increasing maximal response (Roy et al., 2007).

Human C6orf117 was identified as a possible paralog to MRAP (Metherell et al., 2005). This gene was subsequently designated MRAP2. The human MRAP2 gene has four exons, exons 2–4 code for a 205 amino acid residue protein (Chan et al., 2009). MRAP2 is thought to adopt a similar dual topology at the cell surface (Chan et al., 2009). Mouse *Mrap2* gene differs in having two small untranslated exons 1 and 1a (Asai et al., 2013). Zebra fish has a single *Mrap* and two forms of *Mrap2*, designated *mrp2a* and *mrp2b* (Agulleiro et al., 2010). *Mrp2a* and *mrp2b* appear to have different actions and appear at differing time

points in zebrafish development (Sebag et al., 2013). Study of the conservation between the *Mraps* of lampreys, cartilaginous fish, teleosts, and tetrapods has indicated that MRAP2 is the ancestral gene (Webb and Clark, 2010; Dores, 2013).

Tissue Expression of MRAPs

Both MRAP α mRNA and MRAP β mRNA are found in human adrenal tissue, testis, breast tissue, ovary, fat, skin, and jejunum, MRAP α mRNA alone being more widely distributed in digestive tract tissues, the immune system, and in thyroid and pituitary, and MRAP β mRNA alone appearing in brain (Metherell et al., 2005). Evidence for human MRAP mRNA has been detected in the hippocampus, prefrontal cortex, cerebellum, and spinal cord, among other tissues (Gardiner et al., 2002). MRAP expression clearly extends beyond MC2R expression, where expression of MC2R has been reported in the adrenal, bone, adipose tissue, ovaries, testes, skin, and the pituitary (Metherell et al., 2005). MRAP2 mRNA was detected in human adrenal and brain tissue (Chan et al., 2009). In mice *Mrap2* mRNA was detected by RT-PCR in a wider number of tissues including hypothalamus, pons, brainstem, cerebellum, eye, thymus, pituitary, adrenal, gonads, skin, and fat (Asai et al., 2013). A recent report demonstrated that MRAP2 expression in human endometrium was significantly down regulated during endometrial transition from its pre-receptive state to the receptive state (Hu et al., 2014), although the physiological significance of this change is unknown.

MRAPs and Melanocortin Receptors *In vitro*

Several groups have studied the effects of MRAPs on MCR cell surface expression and function in a number of heterologous systems. The absolute requirement of MRAP for the MC2R trafficking and function is clear and recapitulated by many groups (Metherell et al., 2005; Roy et al., 2007; Sebag and Hinkle, 2007). Transient transfection of MRAP α and MRAP2 with MCRs into CHO cells confirmed that MRAP is necessary for surface expression of MC2R and showed that MRAP2 also can enable surface expression of MC2R (Chan et al., 2009). One study using HEK293 cells reported MC2R surface expression in the absence of MRAP (Roy et al., 2007), although this may well have been due to endogenous MRAP2 in some HEK293 cell-lines (Roy et al., 2010). In addition to surface expression, there are suggestions that MRAPs could influence the post-translational glycosylation of MC2R and MC4R (Kay et al., 2013a). MRAP is essential for MC2R to respond to ACTH and although the presence of MRAP2 can enable MC2R to respond to ACTH this is unlikely to be of physiological significance (Gorrigan et al., 2011). The interplay between MRAP and MRAP2 on MC2R function is less clear. Some suggest that MRAP and MRAP2 act in an antagonistic manner whilst others show no effect or an additive effect on MC2R function in the presence of both MRAPs (Chan et al., 2009; Agulleiro et al., 2010; Sebag and Hinkle, 2010).

The reported results on other MCRs vary, which in part may be due to differences in ligands, cell-lines, ratio of MRAP to MCR expressed or be dependent on the ortholog studied. MRAPs have no effect on the trafficking of MC1R and MC3R but reduce

surface expression of MC4R and MC5R (Chan et al., 2009; Sebag and Hinkle, 2009, 2010). In the case of MC5R, MRAP appeared to disrupt MC5R dimerization (Sebag and Hinkle, 2009). Reduced cAMP generation in response to NDP-MSH was demonstrated for human MC1-5R (Chan et al., 2009). Other groups have also shown a significant reduction in MC5R signaling in the presence of MRAPs (Sebag and Hinkle, 2010; Kay et al., 2013b). These studies however found no change in MC4R potency (Sebag and Hinkle, 2010) or an increase in MC4R function (Asai et al., 2013; Sebag et al., 2013). MC4R constitutive activity does appear to be affected in the presence of MRAP/MRAP2 (Asai et al., 2013; Kay et al., 2013b; Sebag et al., 2013). The two isoforms of Mrap2 in zebrafish appears to have differing effects on Mc4r function. Mrap2a inhibits activation of Mc4r whilst Mrap2b suppressed the constitutive activity of the receptor but greatly increased the potency of α MSH (Sebag et al., 2013).

MRAPs and Adrenal Disease

MRAP

Loss-of-function mutations in MRAP give rise to FGD type 2 (Metherell et al., 2005). Patients with FGD type 2 present with symptoms and signs resulting from isolated glucocorticoid deficiency and excess plasma ACTH (Chung et al., 2010). FGD type 2 patients present significantly earlier than those with FGD type 1 individuals harboring MC2R mutations, with the exception of those patients with missense MRAP mutations who present later and with a milder phenotype (Hughes et al., 2010).

In keeping with the importance of MRAP's role in glucocorticoid production, with both MRAP and MC2R are highly expressed in the zona fasciculata. However, the highest levels of expression are found in the undifferentiated zone, believed to contain adrenal stem cells, suggesting that MC2R and MRAP maybe important in adrenal development and/or maintenance (Gorrigan et al., 2011). The adrenal histology from deceased FGD patients would support this notion revealing glomerulosa cell disorganization and loss of fasciculata and reticularis cells (Clark and Weber, 1998).

MRAP is a critical component of the hypothalamic-pituitary-adrenal axis, involved in adrenal responsiveness to ACTH and possibly other adrenal disease processes. In rats, the transcription of Mrap RNA closely tracks the normal ultradian pulses of ACTH and, together with similar patterns of transcription and related protein processing of other components of adrenal steroidogenesis, suggests that Mrap protein availability is closely tied to need for signaling in response to ACTH (Liu et al., 2003).

One study assessed MC2R, MRAP, and MRAP2 expression in human adrenal tissue derived from normal and hyperplastic adrenal gland, and from adrenocortical adenomas and carcinomas (Hofland et al., 2012). Their data suggested that the effect of ACTH stimulation on the expression of the ACTH receptor complex comprising MC2R, MRAP and MRAP2 assists in the production of a functioning complex, although the level of MRAP2 being insufficient to reduce its sensitivity to ACTH.

MRAP2

In vitro, MRAP2 has been shown to enable MC2R trafficking to the cell surface and subsequent signaling. N-linked glycosylation appears critical in this process (Chan et al., 2009). The dose of ACTH required to activate the receptor is however 1000 times higher than that compared to MRAP (Sebag and Hinkle, 2010; Gorrigan et al., 2011), which would explain the inability of MRAP2 to rescue MC2R function in patients with MRAP mutations. Furthermore, significantly lower levels of Mrap2 expression compared with Mrap expression are found in adult rat adrenal gland. Unlike Mrap, which is highly expressed in the zona fasciculata, Mrap2 appears sparsely expressed throughout the adult adrenal cortex (Gorrigan et al., 2011). Although, Mrap2 appears highly expressed in the developing adrenal gland, to date no adrenal phenotype have been described in the Mrap2 KO mice (Gorrigan et al., 2011; Asai et al., 2013).

MRAPs and Obesity

MRAP2

MRAP2 was shown to interact with all MCRs and the expression in the hypothalamus and PVN pointed to a role in central melanocortin regulation of metabolism and appetite (Chan et al., 2009). A recent publication describing obesity in rodents and humans with MRAP2 deficiency has demonstrated that this is indeed the case (Asai et al., 2013). Furthermore, zebrafish deficient of MRAP2 isoforms was shown to have disrupted growth and development supporting the role of MRAP2 in metabolism homeostasis (Sebag et al., 2013).

Phenotypically, global Mrap2 KO mice on an sv129 genetic background fed a chow *ad libitum* diet develop severe obesity at a young age and were found to be significantly heavier than their wild type counterparts at approximately 6 weeks of age. Mrap2^{-/-} mice have increased body length and fat deposits, whilst percentage lean mass was reduced. Heterozygous Mrap2^{+/-} mice have an intermediate phenotype (Asai et al., 2013).

Serum leptin was elevated in Mrap2 null mice, which normalized following weight loss through food restriction. No differences in fasting serum insulin concentration, response to glucose load or serum T3 and T4 levels were detectable between null and wild-type animals, whilst Mrap2^{-/-} males have lower 24 h urine epinephrine and norepinephrine. AgRP mRNA levels in the hypothalamus are increased in Mrap2 null animals without changes in POMC mRNA. Although brown fat deposit was enlarged in obese mice, response to cold challenge normal and Ucp1 mRNA levels in brown fat increased appropriately when mice were subjected to 4°C for 18 h (Asai et al., 2013).

Interestingly, no increase in food intake was detected and despite paired feeding, Mrap2^{-/-} mice became significantly heavier in weight compared with Mrap^{+/+} littermates. Food restriction in Mrap2^{-/-} mice (90% (females) or 87% (males) of wild type intake) was required to normalize their weight gain to that of wild-type Mrap2^{+/+} mice. Faecal energy content was also indistinguishable between Mrap2^{-/-} and Mrap2^{+/+} mice (Asai et al., 2013).

Analysis of young mice prior the divergence of weight between null and wild-type mice demonstrated indistinguishable 24 h energy expenditure and respiratory exchange ratio (RER) when measured by indirect calorimetry. There was no difference in locomotor activity during the day or night and body temperature even when challenged by cold was not different (Asai et al., 2013).

Asai et al. demonstrated that Mrap2 was expressed in several sites of the mouse brain and in the PVN co-localized with Mc4r expressing neurons (Asai et al., 2013), suggestive of an MC4R dependent mechanisms. Mrap2^{-/-} mice share some phenotypic similarities with Mc4r KO mice, which are heavier with increased length and adiposity. Heterozygous Mc4r^{+/-} mice have an intermediate weight phenotype (Huszar et al., 1997). In support of an MC4R mechanism, mice with conditional deletion of Mrap2 in Sim1 neurons that express Mc4r were equally obese and phenotypically similar to global Mrap2^{-/-} mice (Asai et al., 2013). However, differences exist between Mrap2^{-/-} and Mc4r^{-/-} mice. In Mc4r^{-/-} mice, obesity is attributable to hyperphagia and reduced energy expenditure. The Mc4r null mice also have increased lean mass (Balthasar et al., 2005; Sutton et al., 2006).

In humans, disabling mutations of MC4R are the most common cause of monogenic obesity and found in up to 6% of severely obese patients (Vaisse et al., 1998; Yeo et al., 1998). In comparison, genetic screening of two large obese pediatric cohorts identified only four rare heterozygous MRAP2 variants (N88Y, L115V, R125C, E24X) in patients with severe early onset obesity (Asai et al., 2013). The individual carrying the most damaging variant, E24X variant, was the most severely affected with a reported BMI of 63 kg/m² (BMI SDS 4.7) at the age of 19 years.

Data from zebrafish supports the notion that MRAP2 is an MC4R accessory protein capable of regulating the function of MC4R (Asai et al., 2013; Sebag et al., 2013). However, several

lines of evidence from the Mrap2^{-/-} mice data would suggest the possibility of Mc4r independent mechanisms. Firstly, Mrap2^{-/-} mice remain responsive to treatment with MTII, a Mc3r and Mc4r agonist, whilst anorexic response to MTII is abolished in Mc4r^{-/-} mice. Secondly, Mrap2^{-/-} and Mc4r^{-/-} double KOs are less obese compared with Mc4r^{-/-} mice alone and finally the Mrap2^{-/-} does not completely replicate Mc4r^{-/-} mice phenotype.

The phenotype of the Mc3r homozygous knockout mice is of interest. Mc3r^{-/-} animals are not obviously hyperphagic but eventually become heavier than wild type mice on a standard chow diet, and have more adipose tissue and smaller bones, but no significant difference in energy expenditure (Chen et al., 2000; Begriche et al., 2011). Heterozygous Mc3r knockout mice are similar to wild type mice (Chen et al., 2000). More recently it has been shown that MRAP2, but not MRAP, localizes at the apical plasma membrane in the presence of MC3R in a polarized cell, MC3R also localizing at the apical membrane (Park et al., 2014). Receptors and other structures in neurons have precisely determined locations and are associated with dedicated trafficking mechanisms in which MRAPs may have a role.

There is no suggestion that Mrap2 plays a role in MC1R or MC2R function as Mrap2^{-/-} mice have normal coat color and corticosterone production (at baseline and when stressed) (Asai et al., 2013).

MRAP

It is not yet known if MRAP is associated with mammalian obesity. There is a single case report describing a family with a splice site mutation of MRAP. Family members homozygous and heterozygous for the mutation were obese compared with normal weighted unaffected members (Rumie et al., 2007). As yet the knockout mouse has not been reported. In murine 3T3-L1 cells,

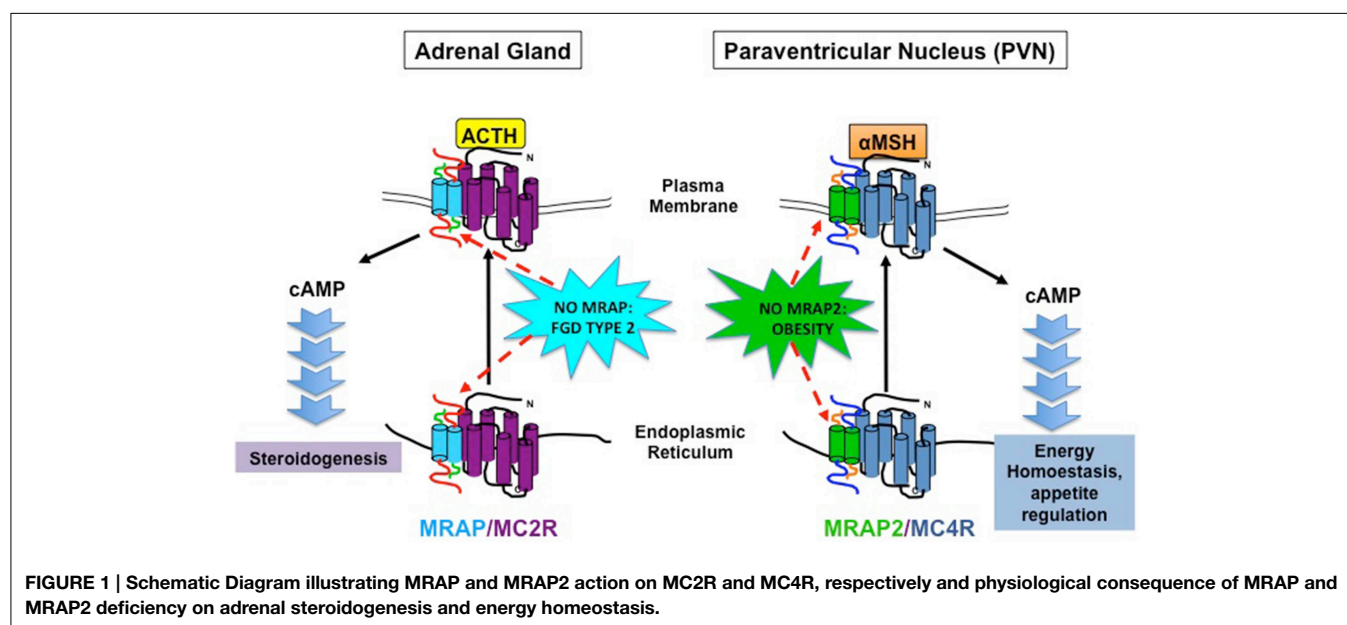


FIGURE 1 | Schematic Diagram illustrating MRAP and MRAP2 action on MC2R and MC4R, respectively and physiological consequence of MRAP and MRAP2 deficiency on adrenal steroidogenesis and energy homeostasis.

ACTH triggers lipolysis and knockdown of Mrap in these cells substantially inhibits lipolysis (Kim et al., 2013). Furthermore, the promoter of Mrap was found to include a region binding the transcription factor PPAR γ that regulates adipogenesis in fibroblasts (Tontonoz et al., 1994; Kim et al., 2013).

Future of MRAPs

The discovery of MRAP and MRAP2 has initiated a paradigm shift in our understanding of MCR and GPCR signaling (Figure 1). For MRAP, the ability to functionally express MC2R in non-adrenal cell lines has opened up many opportunities including screening for MC2R peptide antagonists (Bouw et al., 2014). Moreover, the finding that MRAP2 is associated with mammalian obesity is exciting and could provide a novel therapeutic target at a time when obesity is at epidemic levels. There are many scientific questions yet to be answered. For example, the precise mechanism of how MRAP2 causes obesity

is not fully understood. It is intriguing that no difference in food intake or energy expenditure was detected in Mrap2^{-/-} compared to wild-type littermates. This may represent the lack of sensitivity of the systems in place to detect the relatively small changes in food intake and energy expenditure. If so this demonstrates the fine balance in energy homeostasis, where small changes could tip the scale leading to significant weight gain. However, in light of the differences between Mc4r^{-/-} and Mrap2^{-/-} mice, it is likely that MC4R independent mechanisms as well as MCR independent pathways are at play. The complexity of these proteins and how they regulate MCR and GPCR function is only just beginning to be explored.

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Molecular cloning and characterization of the allatotropin precursor and receptor in the desert locust, *Schistocerca gregaria*

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Allatotropins (ATs) are pleiotropic neuropeptides initially isolated from the tobacco hornworm, *Manduca sexta*. In 2008, the first receptor for AT-like peptides (ATR) was characterized in *Bombyx mori*. Since then, ATRs have also been characterized in *M. sexta*, *Tribolium castaneum*, *Aedes aegypti* and *Bombus terrestris*. These receptors show sequence similarity to vertebrate orexin (ORX) receptors. When generating an EST-database of the desert locust (*Schistocerca gregaria*) central nervous system, we found cDNA sequences encoding the *Schgr*-AT precursor and a fragment of its putative receptor. This receptor cDNA has now been completed and functionally expressed in mammalian cell lines. Activation of this receptor, designated as *Schgr*-ATR, by *Schgr*-AT caused an increase in intracellular calcium ions, as well as cyclic AMP (cAMP), with an EC₅₀ value in the nanomolar range. In addition, the transcript distribution of both the *Schgr*-AT precursor and *Schgr*-ATR was investigated by means of quantitative real-time PCR. Moreover, we found more evidence for the myotropic and allatostimulatory actions of *Schgr*-AT in the desert locust. These data are discussed and situated in a broader context by comparison with literature data on AT and ATR in insects.

Keywords: GPCR, insect, juvenile hormone, neuropeptide, orexin, peptide, motility

Introduction

Allatotropin (AT) was originally identified as an amidated tridecapeptide isolated from the nervous system of the lepidopteran *Manduca sexta*. It was named after its first known biological function, namely the ability to stimulate juvenile hormone (JH) biosynthesis in the *corpora allata* (CA) *in vitro* (Kataoka et al., 1989). Most ATs have a conserved C-terminal pentapeptide that consists of a TARGFa motif although the hymenopteran AT has an exceptional TAYGfA C-terminal (Veenstra et al., 2012). There are also AT-like peptides (ATLs) that contain more variation in their C-terminal motif but they can elicit allatotropic activity as well (Lee et al., 2002). ATs have been isolated from numerous arthropod species, identified from the sequences of cloned genes, or deduced *in silico* from nucleotide sequence databases (Elekonich and Horodyski, 2003; Weaver and Audsley, 2009; Egekwu et al., 2014). Despite its widespread appearance in numerous insects, neither the AT precursor gene nor the AT receptor gene have been identified in *Drosophila melanogaster* or in any

other members of this genus (Hewes and Taghert, 2001; Van-den Broeck, 2001). Related peptides have been reported in other phyla beyond Arthropoda. These were isolated in mollusks (Harada et al., 1993; Li et al., 1993; Veenstra, 2010), flatworms (Adami et al., 2011) and annelids (Ukena et al., 1995; Veenstra, 2011), and recent phylogenetic analysis also showed the presence of this peptidergic system in other protostomes, but not in nematodes, and in some deuterostomes (Mirabeau and Joly, 2013).

AT has pleiotropic functions in a variety of insect species. It stimulates visceral muscle activity (Paemen et al., 1991; Duve et al., 1999, 2000), heart activity (Veenstra et al., 1994), ventral diaphragm oscillation (Koladich et al., 2002), plays a role in the photic entrainment of the circadian clock (Petri et al., 2002), controls the release of digestive enzymes in the midgut (Lwalaba et al., 2010), inhibits active ion transport in the midgut (Lee et al., 1998) and stimulates the secretion of saliva and the contractions of the muscles surrounding the salivary glands (Masood and Orchard, 2014). In *Culex pipiens*, ovarian development was arrested when nondiapausing females were injected with AT dsRNA immediately after adult eclosion, mimicking the diapausing phenotype (Kang et al., 2014). Furthermore, in *T. castaneum*, disrupted adult development and fecundity was observed after injections of AT dsRNA in young pupae (Abdel-latif and Hoffmann, 2014).

It has been suggested that the ancestral role for this peptide family is related to its myotropic role, while the stimulation of JH biosynthesis evolved secondarily in some insect groups (Elekonich and Horodyski, 2003). The myotropic activity of AT on the gut was also suggested to be important for feeding, since gut contractions are necessary to allow food motility and the flow of digestive enzymes (Oeh et al., 2001; Audsley and Weaver, 2009; Nagata et al., 2012).

ATs exert effects on their cellular targets by binding to receptors with high affinity binding sites that are members of the family of rhodopsin-like G protein-coupled receptors (GPCRs). The AT receptors (ATRs) are orthologous to vertebrate orexin/hypocretin receptors. To date, five ATRs have been characterized; namely the neuropeptide 16 receptor in *Bombyx mori* (Yamanaka et al., 2008), the receptors of *M. sexta* (Horodyski et al., 2011), *Tribolium castaneum* (Vuerinckx et al., 2011), *Aedes aegypti* (Nouzova et al., 2012) and *Bombus terrestris* (Verlinden et al., 2013). The first three were dose-dependently activated by *Manse*-AT. The *T. castaneum* receptor was also activated by *Schgr*-AT (which is identical to *Lom*-AG-MT1, the AT from *Locusta migratoria*) and by an endogenous AT-like peptide (*Trica*-ATL) predicted from the *Tribolium* genome (Vuerinckx et al., 2011). The ATR receptor of *B. terrestris* also responded to *Manse*-AT, *Schgr*-AT, and *Trica*-ATL, but much higher concentrations were needed for generating these pharmacological effects (Verlinden et al., 2013). Activation of these AT(L) receptors resulted in an elevation of intracellular calcium and cAMP concentrations (Horodyski et al., 2011; Vuerinckx et al., 2011; Verlinden et al., 2013).

ATR-like receptor genes can also be found in the genomes of the mosquitoes *Anopheles gambiae* and *Culex quinquefasciatus*, the pea aphid *Acyrtosiphon pisum*, the kissing bug *Rhodnius*

prolixus, the monarch butterfly *Danaus plexippus*, the jewel wasp *Nasonia vitripennis*, the honey bee *Apis mellifera*, the alfalfa leaf-cutter bee *Megachile rotundata*; the ant species *Harpegnathos saltator*, *Acromyrmex echinator* and *Solenopsis invicta* and various other insect species (Caers et al., 2012 and unpublished BLAST analysis).

In *Locusta migratoria* a member of the AT family was first identified as the accessory gland myotropin 1, since it was isolated from the male accessory glands and shown to stimulate contractility of the locust oviduct (Paemen et al., 1991, 1992). In *S. gregaria*, AT was found in the brain (protocerebrum, antennal lobes, and tritocerebrum), the circumoesophageal connectives, the suboesophageal ganglion (SOG), the stomatogastric nervous system and all thoracic and abdominal ganglia. No mass peak corresponding to AT was found in the locust *corpora cardiaca* (CC) or retrocerebral complex (Homberg et al., 2004; Clynen and Schoofs, 2009).

We now complement the data obtained in the above mentioned lepidopteran, coleopteran, dipteran and hymenopteran species with a quantitative analysis of the AT precursor and receptor transcripts in different tissues of a representative of the hemimetabolous insects, *S. gregaria*. In addition, we show more evidence for the allatostimulatory and myoactive roles of *Schgr*-AT in the desert locust.

Materials and Methods

Rearing of Animals

Gregarious desert locusts were reared under crowded conditions with controlled temperature ($30 \pm 1^\circ\text{C}$), light (14 h photoperiod) and ambient relative humidity (40–60%). The locusts were kept at high density (>200 locusts/cage) in special wooden cages and fed daily with fresh cabbage leaves supplemented with dry oat flakes. Mature females deposited their eggs in pots filled with a slightly moistened sterile sand mixture (7 parts sand, 3 parts peat, and 1 part water). After oviposition, these pots were collected once a week and set apart in empty cages, resulting in pools of hatched first instar hoppers, which differed by no more than 7 days in age. Depending on the experimental conditions, the locusts were further synchronized at the time of ecdysis (Badisco et al., 2011a; Marchal et al., 2011).

The breeding of solitary desert locusts was performed under isolated conditions according to the method described by Hoste et al. (2002). Newly hatched hoppers were separated at the day of eclosion and were placed in individual containers. Temperature, light-dark photoperiods and food supply were similar for isolated-reared and crowded-reared locusts. All solitary animals came from stocks that were reared under isolated conditions for at least three generations. To characterize the phase status of crowded-reared and isolated-reared locusts, morphometric measurements of femur length (F), caput width (C), and elytra (E) were performed (Dirsch, 1953). The F/C ratio increased, whereas the E/F ratio decreased in successive isolated-reared generations, indicating that individuals shifted toward the solitary phase. The color and behavioral characteristics of crowded- and isolated-reared locusts were very typical for the gregarious and solitary phase, respectively.

Tissue Collection

The locust tissues were dissected under a binocular microscope and immediately snap frozen in liquid nitrogen. In a first experiment, we collected three pools of each tissue (brain, optic lobes, *corpora cardiaca*, *corpora allata*, prothoracic gland, SOG, salivary gland, prothoracic ganglion, mesothoracic ganglion, metathoracic ganglion, gonads, fatbody, flight muscle, foregut, midgut, hindgut, Malpighian tubules, and male accessory gland) of 10 day old gregarious and solitary males and females. The three pools consist of respectively 40, 10, and 10 animals. In a second tissue collection the abdominal ganglia were dissected from 10 day old gregarious animals. The first three (1–3) abdominal ganglia are fused to the metathoracic ganglion and the last four (8–11) are fused to each other and form the terminal abdominal ganglion (Burrows, 1996). Abdominal ganglia 4–5 and 6–7 were dissected together. The three pools males and females each consist of 10 animals. Until further processing, we stored all the tissue samples at -80°C to prevent degradation.

RNA Extraction and cDNA Synthesis

The dissected pooled tissues (<100 mg) were collected in “MagNa Lyser green beads” 2.0 ml tubes (Roche). Semi-automated homogenization of these samples was performed in a MagNa Lyser[®] Instrument (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Total RNA was extracted from the tissue homogenate utilizing an “RNeasy[®] Lipid Tissue Mini Kit” (Qiagen, Germantown, MD) in combination with a DNase treatment (RNase-free DNase set, Qiagen) to eliminate potential genomic DNA contamination.

After verification of the RNA quantity and quality with the Nanodrop (Thermo Fisher Scientific Inc.), we transcribed the resulting total RNA using the SuperScript[®] III Reverse Transcriptase (Invitrogen[™] Life Technologies, Carlsbad, CA) utilizing random hexamers and oligodT’s as described in the protocol. Afterwards, the resulting cDNA was diluted tenfold.

Molecular Cloning

The *Schgr*-AT precursor and a partial fragment of the putative *Schgr*-ATR were found by scanning the EST database of *S. gregaria* (Badisco et al., 2011b). The sequence of the *Schgr*-AT precursor was confirmed by sequencing the plasmid that was used to produce the cDNA library. Additional sequence information of *Schgr*-ATR was obtained by 3’ and/or 5’ rapid amplification of cDNA-ends (RACE) using the “5’/3’ RACE Kit, 2nd Generation” (Roche) in combination with *Schgr*-ATR gene specific primers (see Supplementary Table 1).

cDNA covering the entire *Schgr*-ATR was amplified using a three step procedure. In the first step gene specific cDNA was made using the “Transcriptor High Fidelity cDNA Synthesis Kit” (Roche) and the gene specific primer 5’-TGATAACA TCACTCTGTAT-3’. Next, two PCR rounds were performed using the “Pwo DNA Polymerase” (Roche). The following cycle program was used twice: 94°C for 180 s followed by 30 cycles of 94°C for 45 s, 61°C for 60 s, 72°C for 120 s. The program ended at 4°C after a final elongation at 72°C for 10 min. In the first PCR round, the forward primer: 5’-TCTGCCACAGTACA TCCAA-3’ and the reverse primer: 5’-CACTCCACTAGCGAC

CACAA-3’ were used and in the second PCR round the forward primer: 5’-CACCATGACAGAGAACGAAAC-3’ and the reverse primer: 5’-GTTGCGGGTAAGGAGGTGT-3’ were used. After the first PCR round a PCR clean-up was performed using the “GenElute[™] PCR Clean-Up Kit” (Sigma-Aldrich[®]).

The resulting PCR products were purified from a 1% agarose gel with the “GenElute[™] Gel extraction Kit” (Sigma-Aldrich[®]). The *Schgr*-AT precursor was cloned in a “pCR[™]4-TOPO” vector (Invitrogen[™]) and the *Schgr*-ATR was cloned into a “pcDNA[™]3.1/V5-His TOPO[®]” vector (Invitrogen[™]) following the manufacturer’s instructions. The vector was transformed into One Shot[®] TOP10 chemical competent *E. coli* cells (Invitrogen) and grown on LB agar plates (35 g/l; Sigma-Aldrich[®]) with ampicillin (10 mg/ml; Invitrogen[™]). Colonies with an insert were collected and grown in LB medium (Sigma-Aldrich[®]) with ampicillin (10 mg/ml). The plasmid was purified using the “GenElute[™] Plasmid Miniprep Kit” (Sigma-Aldrich[®]). DNA Sequences were determined using the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems[®]) following the protocol outlined in the “BigDye[®] Terminator v1.1 Cycle Sequencing Kit” (Applied Biosystems[®]).

Phylogenetic and Structural Analysis

We compared the *Schgr*-ATR sequence with other insect ATR(-like) sequences. We aligned the following sequences with Multiple Sequence Comparison by Log-Expectation (MUSCLE; Edgar, 2004): *S. gregaria* ATR (GenBank acc. no. [JN543509](#)), *M. sexta* ATR (GenBank acc. no. [ADX66344](#)), and *T. castaneum* ATR (GenBank acc. no. [XP_973738](#)). In addition, a phylogenetic tree was constructed with the neighbor-joining method, using the amino acid sequences [starting from transmembrane region (TM) 1 and ending with the TM7] from the *Schgr*-ATR, ATR-like receptors of insect organisms [the ones mentioned above, *B. terrestris* ATR (GenBank acc. no. [XP_003402490](#)), the *A. mellifera* ATR (GenBank acc. no. [XP_001120335](#)), *M. rotundata* ATR (GenBank acc. no. [XP_003708421](#)), *N. vitripennis* ATR (GenBank acc. no. [XP_008217710](#)), *R. prolixus* ATR (GenBank acc. no. [AHE41431](#)), *A. aegypti* ATR (GenBank acc. no. [AEN03789](#)), *B. mori* neuropeptide A5 and A16 receptor (GenBank acc. no. [NP_001127740](#) and [NP_001127714](#)), and *D. plexippus* ATR (GenBank acc. no. [EHJ74388](#))] and the FMRamide receptor of *D. melanogaster* (GenBank acc. no. [AAF47700](#)), to root the tree (MEGA software vs. 6; Tamura et al., 2013; 1000-fold bootstrap resampling).

Cell Culture and Transfections

Pharmacological analyses were performed in Chinese hamster ovary (CHO) WTA11 cells stably co-expressing the bioluminescent protein apoaequorin (Brough and Shah, 2009) and the promiscuous $G_{\alpha 16}$ subunit, which couples most agonist-induced GPCRs to the phospholipase C and calcium pathway, irrespective of their natural signaling cascade (Offermans and Simon, 1995; Milligan et al., 1996 cell lines were obtained from the Free University of Brussels and Euroscreen, Belgium). In subsequent experiments, CHO-PAM28 cells stably expressing apoaequorin, but not the promiscuous $G_{\alpha 16}$, and human embryonic kidney

(HEK) 293 cells (Invitrogen™) were used to measure the *Schgr*-ATR downstream signaling properties via calcium and cAMP, respectively.

CHO-WTA11 cells, CHO-PAM28 cells and HEK293 cells were cultured in monolayers in Dulbecco's Modified Eagles Medium nutrient mixture F12-Ham (DMEM/F12) (Sigma-Aldrich®) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich®) to prevent bacterial contamination of gram-positive and gram-negative bacteria, respectively. The medium was also supplemented with 10% fetal bovine serum (Sigma-Aldrich®). For CHO-WTA11 cells, 250 mg/ml zeocin (Invitrogen™) was added to the medium, whereas for CHO-PAM28 cells, 5 µg/ml puromycin (Sigma-Aldrich®) was added to the medium. Puromycin and zeocin were initially used to select for cells stably expressing apoaquorin (CHO-PAM28) (Torfs et al., 2002), or both apoaquorin and $G_{\alpha 16}$ (CHO-WTA11) (Blanpain et al., 1999) and are thus still used as additional antibiotics in the appropriate screens. All cells were maintained in an incubator at 37°C with a constant supply of 5% CO₂.

Transfections with pcDNA™3.1-*Schgr*ATR or empty pcDNA™3.1 vector were carried out in T75 flasks at 60–80% confluency. Transfection medium for CHO cells was prepared using the Lipofectamine LTX Kit (Invitrogen™) with 2.5 ml DMEM/F12, 12.5 µl Plus™ Reagent and 5 µg vector construct (pcDNA™3.1-*Schgr*ATR or empty pcDNA™3.1 vector) in 5 ml polystyrene round-bottom tubes. After a 5 min incubation period at room temperature, 30 µl LTX was added to the medium. After a further incubation period of 30 min at room temperature, the medium was removed from the cells and the DNA/LTX mix was added dropwise to the cells followed by 3 ml of fresh complete medium. The transfection medium used for HEK293 cells was similar to that used for CHO cells except that in addition to 4 µg of pcDNA™3.1-*Schgr*ATR expression construct (or empty pcDNA3.1 vector plasmid), cells were co-transfected with 2 µg of reporter CRE(6×)-Luc plasmid. This reporter plasmid contains six tandem repeats of a cAMP Responsive Element (CRE) in front of a minimum collate promoter and the ORF of luciferase (this reporter plasmid was also used in various other studies: for example Hearn et al., 2002; Johnson et al., 2004). Following transfection, cells were incubated overnight (37°C, 5% CO₂), then 10 ml of cell medium was added followed by a second overnight incubation (37°C, 5% CO₂). Ligand-induced changes in either, intracellular calcium or cAMP were then monitored in the cells as described below.

Calcium Reporter Assay in CHO Cells

CHO cells (CHO-WTA11 or CHO-PAM28) transfected with receptor expression construct (or empty vector) were detached with PBS, complemented with 0.2% EDTA (pH 8.0), and rinsed off the flask with DMEM/F12 without phenolred (Gibco®). The number of viable and nonviable cells was determined using a NucleoCounter® NC-100™ (Chemometric). The cells were pelleted for 4 min at 800 rpm and resuspended to a density of 5×10^6 cells/ml in sterile filtered bovine serum albumin (BSA) medium (DMEM/F12 with L-glutamine and 15 mM HEPES,

without phenolred, supplemented with 0.1% BSA) and loaded with 5 µM Coelenterazine_h (Invitrogen™). Next, the cells were incubated for 4 h in the dark, at room temperature, while gently shaken to reconstitute the holo-enzyme aequorin. After a tenfold dilution 30 min prior to the screening, 50 µl cell solution was injected in the wells (~25000 cells/well) and exposed to *Schgr*-AT (GL Biochem, Shanghai, China) reconstituted in several dilutions in BSA medium distributed in the 96-well plate. In every row, BSA medium without potential ligands was placed in one well to serve as the blank for that row. The calcium response was recorded for 30 s on a multimode microplate reader Mithras LB 940 at a wavelength of 469 nm (Berthold Technologies). After 30 s, 0.1% Triton X-100 was added and the signal was measured for another 10 s. Triton X-100 is a non-ionic surfactant that will break the cell membranes so the total cellular calcium content can be measured. The total calcium signal (ligand + Triton X-100) is representative for the amount of cells present in the well. The response of each blank (small signal caused by cells ruptured by the injection in the wells) was subtracted from the luminescence obtained for wells within the same row. Calculations were made using the output file from the Microwin software (Berthold Technologies) in Excel (Microsoft). Further analysis was done in Excel and GraphPad Prism 5. An illustration of this assay is shown in Supplementary Figure S1.

Cyclic AMP (cAMP) Reporter Assay in HEK293 Cells

To monitor changes in intracellular cAMP levels, HEK293 cells co-transfected with receptor construct (or empty vector) and reporter gene plasmid (CRE₆×-luciferase) were detached with PBS, complemented with 0.2% EDTA (pH 8.0), and rinsed off the flask with DMEM/F12 without phenolred (Gibco®). The number of viable and nonviable cells was determined using the NucleoCounter® NC-100™. The cells were pelleted for 4 min at 800 rpm and finally resuspended to a density of 1×10^6 cells/ml in DMEM/F12 without phenolred, but containing 200 µM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich®; 20 µl of 0.1 M IBMX in DMSO in 10 ml DMEM/F12) to prevent cAMP breakdown. Into each well of a white 96-well plate 50 µl of cell suspension (~50000 cells/well) was added to either 50 µl of DMEM/F-12 (with IBMX, but without phenolred) or DMEM/F-12 [with IBMX, without phenolred and containing 10 µM NKH-477 (a forskolin analog; used to enhance cAMP levels in the cells)] containing various concentrations of the allatotropin peptide (GL Biochem, Shanghai, China). In each row of the plate at least one well with only phenol red-free DMEM/F-12 with IBMX was measured. This well is used to calculate the blank level. The cells were then incubated (37°C, 5% CO₂) for 3–4 h. Hereafter 100 µl of steadylite plus™ substrate (PerkinElmer) was added to each well using a multichannel pipette and the plate was incubated for 15 min in the dark. Finally, light emission resulting from the luciferase enzymatic activity was recorded at 0 s and 5 s on a multimode microplate reader Mithras LB 940 at a wavelength of 469 nm. The signal of the two measurements (0 and 5 s) was almost identical; an average of these two was used for further analysis. Results

were analyzed by using Excel and Graphpad Prism 5 Software. An illustration of this assay is shown in Supplementary Figure S2.

qRT-PCR Study of Transcript Levels

Accurate normalization of the raw data was obtained by using the optimal combination of endogenous control genes. The best combination of reference genes was determined using GeNorm (Vandesompele et al., 2002) as described by Van Hiel et al. (2009) and Verlinden et al. (2010). The PCR reactions were performed in a 20 μ l reaction volume following the manufacturer's instructions for the Fast SYBR[®] Green Master Mix (2 \times) (Applied Biosystems[®]). The final concentration of the primers was 500 nM. Primers for the endogenous controls, as well as for the *Schgr*-ATR and *Schgr*-AT target genes, were designed by means of the Primer Express[®] Software v2.0 (Applied Biosystems[®]). For primer sequences, see **Table 1**.

To identify efficient primers for the qRT-PCR, relative standard curves for the endogenous controls and the *Schgr*-ATR and *Schgr*-AT transcripts were generated with serial (10 \times) dilutions of a brain cDNA sample. Reactions were run in duplicate on a StepOne[™] Plus System (ABI Prism, Applied Biosystems[®]) using the following thermal cycling profile: 95°C for 10 min, followed by 40 steps of 95°C for 3 s and 60°C for 30 s. After 40 cycles, samples were run for the dissociation protocol (i.e., melting curve analysis). Analysis of the dissociation curves of the different amplification products revealed a single melting peak. In addition, we analyzed the PCR products via agarose gel electrophoresis, showing the presence of a single band of the expected size for each transcript. Furthermore, sequencing of the PCR products ultimately confirmed the identity of the amplified DNA with their respective target sequences.

To study the transcript levels, we normalized, for each sample, the relative amount of transcript to the endogenous controls (Actin and GADPH) and calculated transcript levels relative to a calibrator sample (in this case, a mix of all measured tissues of males and females, gregarious and solitary). The tissue, phase and sex distribution experiments were repeated three times with independent biological pools of adult *S. gregaria* tissues (40, 10, and 10 animals per pool). We detected no amplification of the fluorescent signal in any negative control sample, proving that the extraction procedure, including the DNase treatment,

effectively removed genomic DNA from all the RNA samples and that there was no contamination. Statistical analysis was performed by means of SPSS (v17.0, SPSS Inc., Chicago, Illinois), using the Mann-Whitney U test for comparing two independent groups. A level of $P < 0.05$ was considered significant.

In a second qRT-PCR we studied the transcript levels in the abdominal ganglia in males and females of gregarious animals. The first three abdominal ganglia are fused to the metathoracic ganglion hence the same samples as in the first transcript study were used. For each sample the relative transcript amounts were normalized to the housekeeping genes coding for Actin and GADPH. The brain sample of the females (a sample from the first tissue distribution) was used as the calibrator sample.

Schgr-AT Bioassay

Schgr-AT was tested on an isolated gut preparation, as described by Schoofs et al. (1990). The midgut from a sexually mature male was ligated at both ends with strings by which the gut was suspended between the arm of a transducer and the bottom of a plastic chamber containing 2.5 ml *S. gregaria* saline (1L: 8.766 g NaCl; 0.188 g CaCl₂; 0.746 g KCl; 0.407 g MgCl₂; 0.336 g NaHCO₃; 30.807 g sucrose; 1.892 g trehalose; pH 7.2) at room temperature (Supplementary Figure S3). The transducer monitored the contractions of the gut, which were visualized on a connected recorder (LKB 2210 recorder). When a constant rhythm of contractions was reached, 25 μ l of 10 mM *Schgr*-AT (GL Biochem, Shanghai, China) dissolved in saline (to reach a final concentration of one micromolar) or the same volume of saline without peptide was added to the chamber. In between two measurements the chamber was rinsed three times with saline; after this the constant contraction rhythm was restored.

In Vitro Measurement of JH

Biosynthesis—Radiochemical Assay (RCA)

Rates of JH release and the JH content were measured by the *in vitro* radiochemical assay (RCA) originally described by Tobe and Pratt (Pratt and Tobe, 1974; Tobe and Pratt, 1974) and further discussed by Feyereisen and Tobe (1981) and Yagi and Tobe (2001). The RCA measures the rate of incorporation of the methyl group from [Methyl-14C] methionine (50 μ M, 2.11 GBq/mmol, New England Nuclear Co.) into JH in isolated CA. CA were dissected out of the head of vitellogenic adult females, since it is known that their CA produce a high amount of JH (Tobe and Pratt, 1975). The dissected CA were directly transferred to conical glass vials holding 50 μ l of radioactive TC199 medium [3 μ Ci/ml medium; lacking L-methionine, glucose, acetate and calcium (Gibco[®], with Hank's salts, HEPES 25 mM)]. The individual CA were shaken at 30°C during the first incubated period of 3 h. Next, CA were transferred to fresh radioactive TC199 medium supplemented with 30 μ M farnesoic acid (FA) to stimulate JH synthesis. 1 μ M *Schgr*-AT was added to the experimental CA. 8 control CA and 9 *Schgr*-AT treated CA were tested. Incubation medium was extracted using 300 μ l of iso-octane. The samples were vortexed and centrifuged for 10 min at 2000 rpm. The top 200 μ l of the iso-octane layer was removed and put into scintillation vials containing 3 ml of

TABLE 1 | Oligonucleotide primers for qRT-PCR used in this study.

| | Forward primer | Reverse primer |
|-------------------|-------------------------------------|---------------------------------|
| Actin | 5'-AATTACCATTTGGTA ACGAGCGATT-3' | 5'-TGCTTCCATAC CCAGGAATGA-3' |
| GAPDH | 5'-GTCTGATGAC AACAGTGCAT-3' | 5'-GTCCATCACGCC ACAACCTTC-3' |
| <i>Schgr</i> -AT | 5'-ATGCAGAACA ACCCGGAAC-3' | 5'-CTGGTTAGCGT CCACGAACCT-3' |
| <i>Schgr</i> -ATR | 5'-CGTCAACCCAGT GATCTACAACCT-3' | 5'-TAGGCGCAC GTCCAGAACA-3' |

Abbreviations used: GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; *Schgr*, *Schistocerca gregaria*.

scintillant (ICN) and measured in a liquid scintillation counter (Beckman, LS-6500).

The effect of *Schgr*-AT on the JH production of the CA was calculated by dividing the difference of the JH production during the second incubation and the JH production during the first incubation by the JH production during the second incubation. Significance was determined with a student's *t*-test in GraphPad Prism 5.

Results

Cloning and Sequence Analysis

As described in the *S. gregaria* EST paper (Badisco et al., 2011b), a partial fragment of an orexin 2 receptor-like/ ATR-like receptor and the *Schgr*-AT precursor are represented in the EST database. These sequences were confirmed by PCR, cloning, and sequencing. We further completed the sequence of the *Schgr*-ATR by rapid amplification of cDNA ends (RACE). The *Schgr*-ATR amino acid sequence is shown in **Figure 1**. The receptor belongs to the rhodopsin-like GPCRs and contains seven transmembrane domains [analyzed with a hidden Markov model for

predicting transmembrane regions (Sonnhammer et al., 1998; Krogh et al., 2001)]. The sequence of the precursor *Schgr*-AT is displayed in **Figure 2**. The sequence contains a signal peptide predicted by SignalP 4.1 (Petersen et al., 2011) and two recognition motifs for proteolytic processing of the preproallatotropin. The G-residue at the C-terminal may be a substrate for peptidyl amidating monooxygenase (PAM) resulting in an amidated neuropeptide (Rouillé et al., 1995; Veenstra, 2000; Veenstra et al., 2012). The obtained nucleotide sequence of the *Schgr*-ATR fragment and the sequence of *Schgr*-AT have been submitted to the European Bioinformatics Institute (EBI) database (*Schgr*-ATR: GenBank accession no. [JN543509](#); *Schgr*-AT: GenBank accession no. [KP233881](#)).

Analysis of Phylogenetic Relationships

Amino acid sequence comparisons between the *Schgr*-ATR and other insect ATR-like receptors show high overall amino acid similarity (identical and conservatively substituted residues; **Figure 1**). The amino acid sequences (TM1-7) of the ATR-like receptors and the FMRamide receptor from *D. melanogaster*,

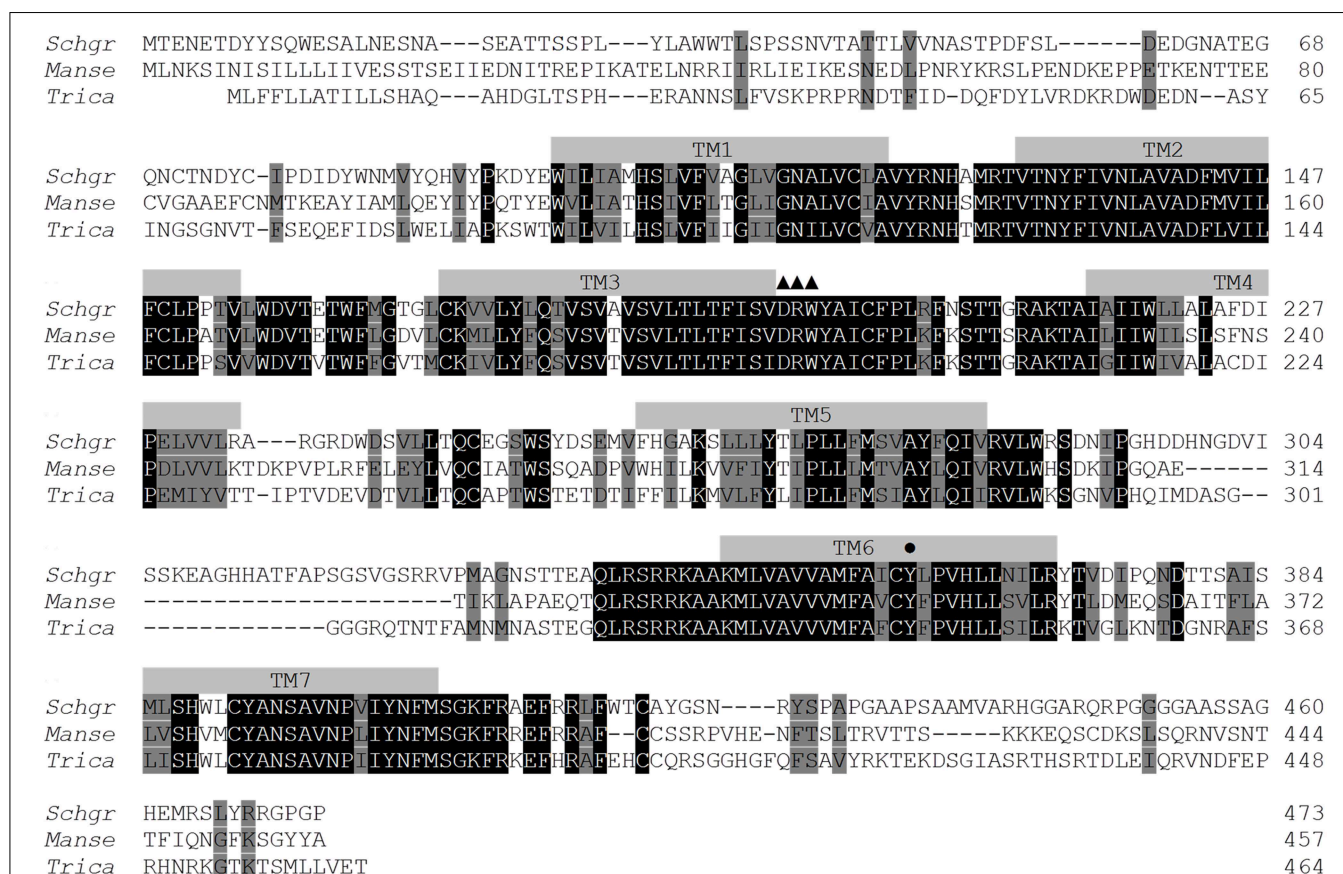


FIGURE 1 | Amino acid sequence of the *Schgr*-ATR (GenBank acc. no. [JN543509](#)) and homologous receptors from *Manduca sexta* (GenBank acc. no. [ADX66344](#)) and *Tribolium castaneum* (GenBank acc. no. [XP_973738](#)). The amino acid position is indicated at the right. Identical residues between the aligned sequences are highlighted in black,

and conservatively substituted residues in gray. Dashes indicate gaps that were introduced to maximize homologies. Putative transmembrane regions (TM1-TM7) are indicated by gray bars. The position of the W (here changed to Y) (•) that is usually conserved in many rhodopsin-like GPCRs and the DRW motif (▲▲▲) are labeled.

| | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|-----|-----|
| ATG | CGC | TGC | GCC | GCC | GCC | GCC | CTG | TGC | CTG | CTG | GTC | GCC | CTC | GCC |
| M | R | C | A | A | A | A | L | C | L | L | V | A | L | A |
| GCC | CTC | TGC | GCC | GCC | GCC | GCC | GCC | GCC | CCC | GCG | GCT | CAC | TAC | GGC |
| A | L | C | A | A | A | A | A | A | P | A | A | H | Y | G |
| CGC | GGC | TCC | CGC | CCC | CGC | ACG | ATA | CGG | GGC | TTC | AAG | AAC | GTG | GCA |
| R | G | S | R | P | R | T | I | R | G | F | K | N | V | A |
| CTC | TCC | ACC | GCG | CGG | GGC | TTC | GGC | AAG | CGA | GAC | GGC | AAC | CAG | CTG |
| L | S | T | A | R | G | F | G | K | R | D | G | N | Q | L |
| GAG | GCC | GCG | CTC | GCT | GAC | CGC | GAC | ACC | ACC | CTC | CCG | GAC | AGC | TTC |
| E | A | A | L | A | D | R | D | T | T | L | P | D | S | F |
| CCT | GTG | GAA | TGG | TTC | GCC | GCC | GAG | ATG | CAG | AAC | AAC | CCG | GAA | CTG |
| P | V | E | W | F | A | A | E | M | Q | N | N | P | E | L |
| GCT | CGC | ATG | ATC | GTC | AGC | AAG | TTC | GTG | GAC | GCT | AAC | CAG | GAT | GGA |
| A | R | M | I | V | S | K | F | V | D | A | N | Q | D | G |
| GAA | CTG | ACG | GCA | GAG | GAA | CTC | CTC | AGG | CCC | ACT | TAC | TGA | | |
| E | L | T | A | E | E | L | L | R | P | T | Y | STOP | | |

FIGURE 2 | Precursor sequence of *Schgr-AT*. The sequence of AT is highlighted in green. The predicted signal peptide sequence is shown in orange and the recognition sites for proteolytic

processing of the proneuropeptide are shown in blue. The G-residue predicted to be transformed into the C-terminal amide is shown in yellow.

to root the tree, were aligned with MUSCLE. A neighbor-joining tree was constructed using MEGA software with 1000-fold bootstrap resampling (Figure 3). The ATRs cluster together as compared to the root of the tree and the lepidopteran and hymenopteran ATRs cluster within their insect class. The overall insect phylogeny is however not respected in the tree. Bootstrap values already indicate that the power of some nodes is less as compared to the lepidopteran and hymenopteran cluster. Future characterization projects will hopefully result in more ATR sequences from diverse phylogenetic classes and will hopefully increase the overall power of phylogenetic studies.

Functional Activation of *Schgr-ATR* with AT

The *Schgr-ATR* was expressed in CHO-WTA11 cells, which express the promiscuous $G_{\alpha 16}$ protein that can induce a calcium rise if an agonist (in this case *Schgr-AT*) binds to the receptor. *Schgr-AT* elicits a sigmoidal dose-dependent response with an EC_{50} value of 4.43×10^{-9} M (Figure 4A; $\log EC_{50} = -8.354 \pm 0.025$, mean \pm SEM). To test if the receptor can induce a calcium rise, the receptor is expressed in CHO-PAM28 cells, which do not express the promiscuous $G_{\alpha 16}$ protein. *Schgr-AT* clearly induced an intracellular calcium response with an EC_{50} value of 5.57×10^{-9} M (Figure 4B; $\log EC_{50} = -8.254 \pm 0.067$, mean \pm SEM). HEK293 cells were used to test whether the receptor can also signal through cAMP. NKH-477 (a forskolin-analog), which activates adenylyl cyclase, will be responsible for an increase of intracellular cAMP levels. If the receptor couples negatively to adenylyl cyclase, a reduction of intracellular cAMP levels would be expected following administration of *Schgr-AT*. This was not observed when *Schgr-ATR* was expressed in the HEK293 cells (results not shown). However, in the absence of NKH-477, a specific increase of luciferase reporter activity was observed with an EC_{50} value of 8.10×10^{-8} M (Figure 4C; $\log EC_{50} = -7.09 \pm$

0.123, mean \pm SEM). CHO-WTA11, CHO-PAM28 and HEK293 cells transfected with an empty pcDNA 3.1 vector did not show any response to *Schgr-AT* (results not shown).

Transcript Level Studies

The expression of the *Schgr-AT* precursor is largely restricted to the central nervous system (Figure 5). No significant differences were observed between samples of gregarious and solitary animals, hence they are represented together. Females in general show higher *Schgr-AT* precursor transcript levels as compared to males. The effect is significant ($p < 0.05$) in the central brain parts, the optic lobes and abdominal ganglia 4-5 and 6-7. Only in the last abdominal ganglion the transcript levels are higher in the males than in the females ($p < 0.05$).

The *Schgr-ATR* is also mainly expressed in the CNS. The highest transcript levels can be measured in the brain, the optic lobes, the metathoracic ganglion and the abdominal ganglia (Figure 5). The receptor also shows relatively high transcript levels in the Malpighian tubules, intestine, male accessory glands, mesothoracic ganglion, prothoracic ganglion, fat body, gonads, CA and the salivary glands. No significant differences were observed between sexes, or phases. Nor did we observe significant differences between the transcript levels in larval and adult CA (results not shown). The *Schgr-ATR* transcript levels are 200–1000-fold lower in the central nervous system as compared to the *Schgr-AT* precursor transcript levels.

Gut Motility Bio-Assay

Schgr-AT was added to the midgut preparation *in vitro* when a constant contraction rhythm was observed. This led to an immediate tetanus (Figure 6A left). After rinsing, the tetanus disappeared and normal contraction rhythm was restored (results not shown). No change in contraction strength or rhythm of the

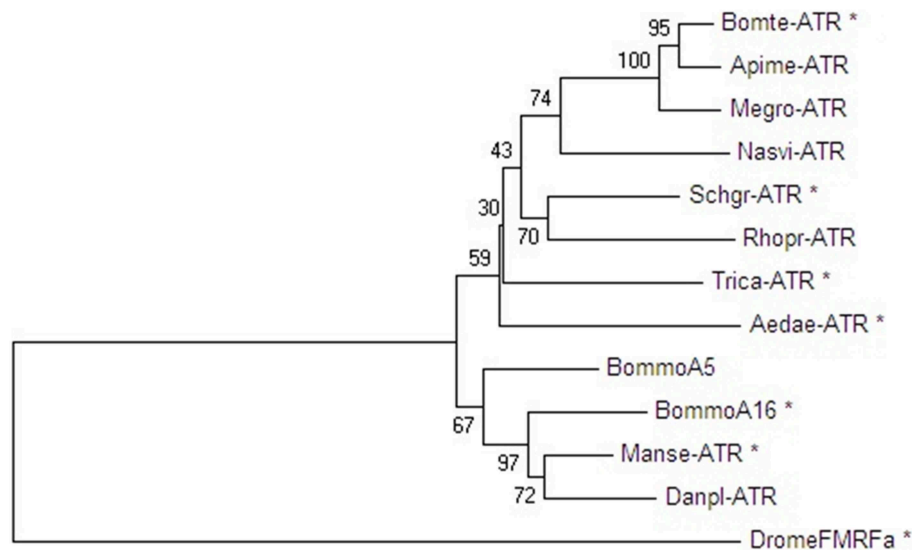


FIGURE 3 | Neighbor-joining tree of insect ATR-like receptors in dendrogram display with representative branch length. Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 6. The FMRamide-receptor of *D. melanogaster* (GenBank acc. no. AAF47700) was used as an outgroup to root the tree. Proteins marked with an asterisk were functionally characterized. Bootstrap-support values are based on 1000 replicates and are indicated on the nodes. The other

GenBank accession numbers are: *S. gregaria* ATR (JN543509), *M. sexta* ATR (ADX66344), *T. castaneum* ATR (XP_973738), *B. terrestris* ATR (XP_003402490), *A. mellifera* ATR (XP_001120335), *M. rotundata* ATR (XP_003708421), *N. vitripennis* ATR (XP_008217710), *R. prolixus* ATR (AHE41431), *A. aegypti* ATR (AEN03789), *B. mori* neuropeptide A5 and A16 receptor (NP_001127740 and NP_001127714), and *D. plexippus* ATR (EHJ74388).

midgut was observed when we added saline without the peptide (results not shown). The entire procedure was repeated and again only change in contraction of the midgut could be observed when adding *Schgr*-AT (Figure 6B right).

In Vitro Measurement of JH Biosynthesis—Radiochemical Assay (RCA)

In the control CA the JH production was slightly lower during the second incubation period when compared to the first incubation. This is likely the result of the natural decrease in JH biosynthesis by senescence of the CA cells or a decrease in JH precursor pools as in the *in vitro* nature of the experiment. However, if the CA were treated with AT during the second incubation period, the JH production increased significantly ($p < 0.05$; Figure 7).

Discussion

Molecular Cloning and Phylogeny

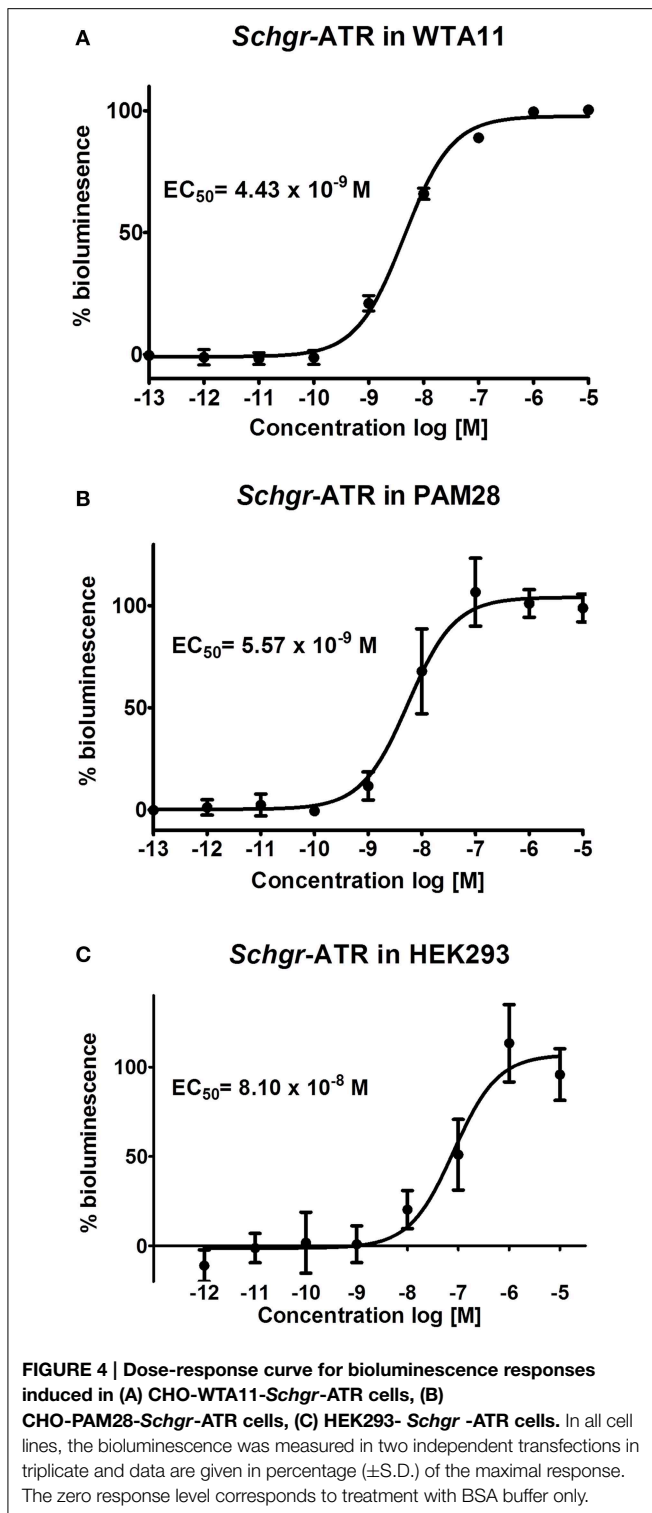
In the present study, we have characterized *Schgr*-ATR, an AT receptor of the desert locust, *S. gregaria*. The obtained sequence has considerable similarity with orthologous receptors from other insects (Figure 1) (Horodyski et al., 2011; Vuerinckx et al., 2011; Nouzova et al., 2012; Verlinden et al., 2013). The sequence contains a DRW (instead of the typical DRY) motif in the second intracellular loop, as it is the case for other AT receptors. AT and its receptor seem to be present in both hemimetabolous and holometabolous insect species (Figure 3), although exceptions exist. *Drosophila* appears to lack both the ligand (Hewes and Taghert, 2001; Vanden Broeck, 2001) and its receptor. It

was first thought that the hymenopteran insects, *A. mellifera* (Hummon et al., 2006) and *N. vitripennis* (Hauser et al., 2010) also lacked an AT-like peptide. However, after a more thorough search, a hymenopteran AT gene was found (Veenstra et al., 2012) and, meanwhile, an AT receptor was characterized in the hymenopteran species *B. terrestris* (Verlinden et al., 2013).

The ATRs show large sequence similarity to the mammalian orexin (ORX) receptors. Although the ORX and AT peptides do not display any obvious sequence similarity and ORX does not activate the invertebrate receptors (Vuerinckx et al., 2011), Mirabeau and Joly found evidence for a common origin of the AT and ORX precursor genes (Mirabeau and Joly, 2013).

Functional Receptor Characterization

We demonstrated that *Schgr*-AT activates the *Schgr*-ATR *in vitro* and causes an increase in both intracellular calcium ion and cAMP levels with an EC_{50} value in the nanomolar range (Figure 4). The higher standard deviation measured in the HEK293 cells compared to the CHO cells can be explained by the absence of a normalization step for the total amount of cells per well, which was used in the calcium reporter assays. The measurement of the bioluminescence in the cAMP reporter assay is dependent on a CRE and thus dependent on the phosphorylation of CREB (cAMP Responsive Element Binding protein). Therefore, it has been speculated that a change in bioluminescence may also be caused by calcium, since CREB can also be phosphorylated by calcium/calmodulin-dependent protein kinase (Johannessen et al., 2004). However, an earlier study of another neuropeptide receptor (*Schgr*-sNPFR) that makes use



of the same assays, showed increased intracellular calcium levels (CHO cell screen), but no increase in bioluminescence in the HEK293 cell screen, which would be expected if the calcium was responsible for the increase in bioluminescence in this screen (Dillen et al., 2013). In addition, the reporter plasmid was also

used in various other studies (for example Hearn et al., 2002; Johnson et al., 2004). These facts suggest that an increase in bioluminescence in this assay is caused directly by an increase in intracellular cAMP levels and not (indirectly) by the increase in intracellular calcium levels.

Our pharmacological data correspond well with previous pharmacological characterizations of ATRs in other insects. Upon activation by their endogenous AT, the AT(L)Rs of *M. sexta*, *T. castaneum* and *B. terrestris* also stimulate intracellular calcium and cAMP levels, although lower EC₅₀ values were measured (Horodyski et al., 2011; Vuerinckx et al., 2011; Verlinden et al., 2013). The fact that the EC₅₀ value of the receptor expressed in HEK293 cells is higher than the EC₅₀ value of the receptor expressed in CHO-PAM28 cells might indicate that the calcium response of *Schgr*-ATR is more sensitive than the cAMP response, although the difference may (in part) be explained by the use of different assays.

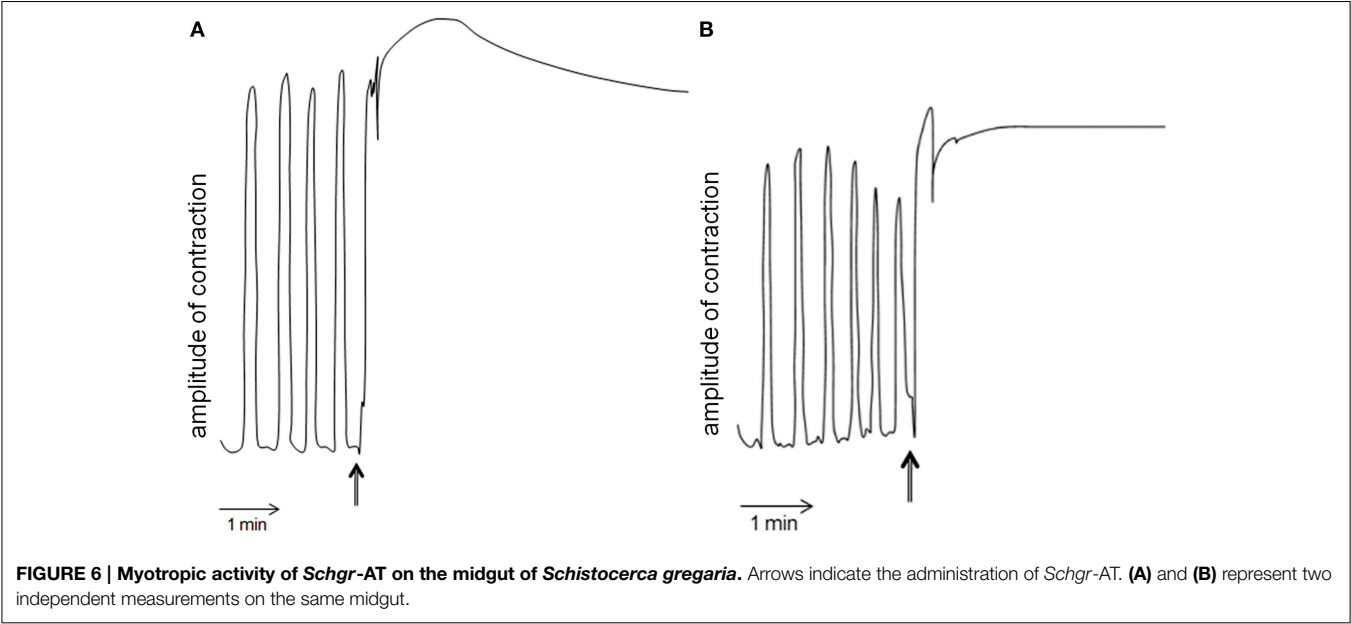
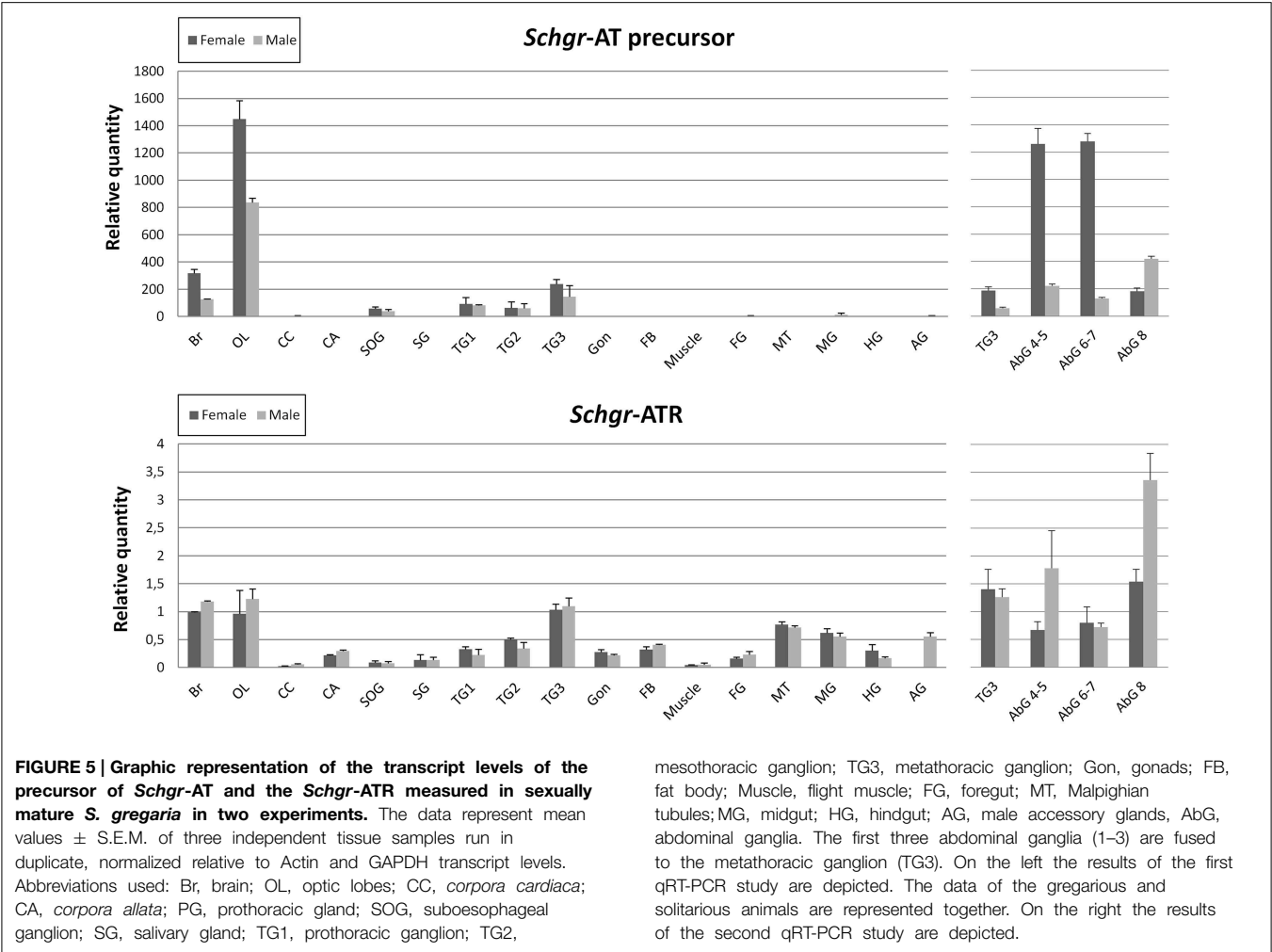
Tissue Distribution and Functions of Allatotropin

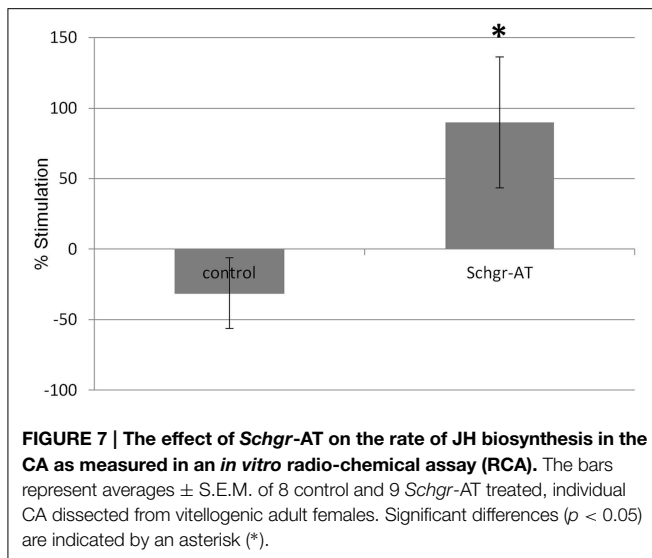
Schgr-AT precursor expression seems to be largely restricted to the central nervous system (Figure 5). This is as expected, since neuropeptides are usually produced in the nervous system and transported toward their target tissues (Caers et al., 2012). Our qRT-PCR data correspond very well with previous immunological and mass spectrometry data obtained in *L. migratoria* (Paemen et al., 1992) and *S. gregaria* (Homberg et al., 2004; Clynen and Schoofs, 2009) confirming the presence of this neuropeptide in extensive areas of the locust brain, including all neuropils in the optic lobe, the antennal lobes, and most areas in the protocerebrum. The first “AT-related” peptide in locusts was originally purified from male accessory glands of *L. migratoria* (*Lom*-AG-MT1) (Paemen et al., 1991) and is identical to *Schgr*-AT. The AT present in the male accessory glands (which show very low relative expression levels) is presumably originating from the (last) abdominal ganglia. Moreover, in other insect species specific neuroendocrine cells in the abdominal ganglia appear to be the most abundant source of AT (Veenstra et al., 1994, 2012; Veenstra and Costes, 1999; Rudwall et al., 2000; Neupert et al., 2009).

The *Schgr*-AT precursor shows 100–1000 fold higher transcript levels in the central nervous system as compared to the *Schgr*-ATR. This can be explained by the fact that neuropeptides are released in large quantities into the periphery, where they will bind to their receptors, to execute their functions. Moreover, the half-life of a peptide is expected to be shorter than the turnover rate of receptors.

The high abundance of ATR in the central nervous systems suggests a critical role in sensory processing, learning and memory and motor control (Elekonich and Horodyski, 2003). In *Leucophaea maderae*, injections of AT near the accessory medulla, which is identified as the location of the circadian clock in this cockroach and part of the optic lobes, resulted in changes in circadian locomotor activity (Petri et al., 2002). Also in *S. gregaria* high expression levels of ATR were measured in the optic lobes.

The ATR expression in the CA is probably related to the stimulatory role of AT on the biosynthesis and release of JH. Already,





Tobe et al. (1977) demonstrated that an allatostimulatory factor released by the CA was responsible for the production of JH. We now also confirmed that *Schgr*-AT indeed stimulates the JH production in the CA (Figure 7). This may also explain why the AT precursor expression is significantly higher in 10 day old adult females as compared to males (Figure 5), since JH (regulated by AT) was demonstrated to be important in females of this age for vitellogenin production and oocyte growth (Pratt and Tobe, 1974; Sevala et al., 1995; Glinka and Wyatt, 1996; Wyatt et al., 1996). The difference was especially pronounced in the abdominal ganglia, hence they could be responsible for activation of the CA.

The high expression levels of *Schgr*-ATR in the Malpighian tubules were also observed in *M. sexta* and suggest that AT may have a role in osmoregulation (Horodyski et al., 2011). The expression of the *Schgr*-ATR in the salivary glands can be related to a role in the stimulation of saliva secretion as was recently discovered in *R. prolixus* (Masood and Orchard, 2014). High transcript levels of *Schgr*-ATR were measured in the digestive system as well. This can be explained by the fact that AT affects the intestinal motility, as was shown with the bio-assay (Figure 6). Another link that can be made with the digestive system, is the impact of the nutritional status on the transcript levels of the AT precursor. In larvae of *M. sexta* and the armyworm *Mythimna separate* it was shown that starvation led to higher transcript levels of AT (Lee and Horodyski, 2002,

2006; Zhang et al., 2008). In larvae, starvation causes an additional molt, indicating that JH levels are elevated. This gives the larvae the opportunity to acquire additional nutrients once they become available in order to successfully complete development to a robust reproductive adult. In contrast, starvation of some insects during the adult stage inhibited oocyte maturation as a consequence of decreasing JH biosynthesis (Tobe and Chapman, 1979; Zhang et al., 2008). The overall regulation of JH titer is complex, since the CA can be influenced by multiple stimulatory and inhibitory factors, and since JH catabolism and binding to JH transport proteins also plays a major role in the control of JH titer (Lee and Horodyski, 2006).

Author Contributions

Molecular cloning and EST database analyses were performed by LB, PV, HV and EL. Functional tests with AT were performed by PV, HV, CL, RV and SZ. The radio chemical assay was performed by EM and ST. The dissections were performed by EM, LB and HV. The pharmacological characterization was performed by EL. Guidance of the study, writing and correction of the manuscript were performed by HV, EL and JV.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fnins.2015.00084/abstract>

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Gut melatonin in vertebrates: chronobiology and physiology

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Melatonin, following discovery in the bovine pineal gland, has been detected in several extra-pineal sources including gastrointestinal tract or gut. Arylalkylamine *N*-acetyltransferase (AANAT) is the key regulator of its biosynthesis. Melatonin in pineal is rhythmically produced with a nocturnal peak in synchronization with environmental light–dark cycle. A recent study on carp reported first that melatonin levels and intensity of a ~23 kDa AANAT protein in each gut segment also exhibit significant daily variations but, unlike pineal, show a peak at midday in all seasons. Extensive experimental studies ruled out direct role of light–dark conditions in determining temporal pattern of gut melatoninergic system in carp, and opened up possible role of environmental non-photocue(s) as its synchronizer. Based on mammalian findings, physiological significance of gut-derived melatonin also appears unique because its actions at local levels sharing paracrine and/or autocrine functions have been emphasized. The purpose of this mini review is to summarize the existing data on the chronobiology and physiology of gut melatonin and to emphasize their relation with the same hormone derived in the pineal in vertebrates including fish.

Keywords: gut melatonin, AANAT, chronobiology, physiology

Introduction

Extensive research carried out in past 50 years have clearly depicted melatonin (5-methoxy-*N*-acetyltryptamine) as a potent chronobiotic molecule involved in the regulation of a variety of physiological functions (1). Following discovery in the bovine pineal gland (2), melatonin is detected in several non-pineal tissues/organs, such as retina, Harderian gland, and gastrointestinal tract (GIT) or gut. However, existing knowledge on melatonin has stemmed largely from the studies on pineal that too in mammals, leaving non-pineal tissues, especially in lower vertebrates, as an interesting topic of research. Though functional characterization of melatonin in gut, relative to that in retina and Harderian gland, has received serious attention, the studies are limited mostly to the mammals, especially rodents and pigs. Moreover, current knowledge on the cellular localization, distribution, and temporal pattern of gut melatonin is scarce and inconsistent as well. Thus, a brief review of the existing literature on gut melatonin appears meaningful for understanding its unique features in vertebrates.

Cellular Localization and Distribution

Localization of melatonin in the enterochromaffin cells (EC) of digestive mucosa of rat was followed by its quantitative estimation in gut tissues. At the sub-cellular level, strongest binding was noted in nuclear fraction, followed by microsomal, mitochondrial, and cytosolic fractions (3).

In mammals, melatonin-producing cells were found in submucosa and muscularis layer of esophagus, the glandular portion of the gastric wall and in the area of Lieberkühn's crypts and Brunner's glands of duodenum, and more specifically in EC of mucosal layer (3). As in birds and mammals, maximum immunoreactivity was noted at the outer margin of lamina propria in mucosal layer of intestinal villi in carp gut (4).

Generally, melatonin concentrations in gut tissues surpass the levels of melatonin in circulation by 10–100 times (3). Day-time levels of gut melatonin were measured in several species (5) of fish, e.g., sturgeon, rainbow trout, and carp (stomach ~102 pg/g, proximal gut or PG ~146 pg/g, and distal gut or DG ~141 pg/g); amphibians, e.g., axolotl (stomach and PG ~44 pg/g and DG ~92 pg/g), and bullfrog (esophagus ~73 pg/g, stomach ~78 pg/g, PG ~20 pg/g, and DG ~152 pg/g); and reptiles, e.g., red-sided garter snake (stomach ~1018 pg/g, PG ~328 pg/g, and DG ~511 pg/g). The midday values of melatonin in the anterior (~550 pg/g), middle (~538 pg/g), and posterior (~578 pg/g) segments of gut in a day-active carp (6) did not indicate any regional variations in its distribution in the same animal, but depicted species-specific variations in a particular gut segment (5). However, it remains obscure whether gut melatonin levels vary between the day-active and night-active animals.

Biosynthesis of Melatonin

The synthesis of melatonin in all the melatonin-synthesizing cells is a four-step phenomenon. First, the precursor L-tryptophan is taken up from the circulation (blood) and is converted to 5-hydroxy-tryptophan (5-HTP) in the mitochondria by Trp-5-mono-oxygenase/hydroxylase and is then decarboxylated in the cytosol by L-aromatic amino acid decarboxylase to form serotonin (5-hydroxytryptamine, 5-HT), that in turn is acetylated (*N*-acetylation) into *N*-acetyl serotonin by arylalkylamine-*N*-acetyltransferase (AANAT) (7), which is considered as the rate-limiting enzyme in melatonin biosynthetic pathway. Finally, *N*-acetyl serotonin is O-methylated by hydroxyindole-O-methyltransferase (HIOMT) to form melatonin (8).

Endogenous melatonin biosynthesis within the ECs of the digestive mucosa has been evident from the studies on the expression of genes for two key melatonin-synthesizing enzymes. The study of gut tissues detected mRNA expression of *Aanat* in rat (9) and *Hiomt* in quail (10) as well as goldfish (11). *Aanat*-2 expression, as noted in the gut of goldfish (11) and rainbow trout (12), was supported by densitometric analysis of AANAT protein in the carp gut (6). The study detected a ~23 kDa AANAT protein corresponding to AANAT in the pineal of pike, trout, and carp (13) in gut tissue homogenates and thereby ensured endogenous synthesis of melatonin in gut.

Chronobiology

Temporal Pattern of Gut Melatonin

Circulating profiles of melatonin in different vertebrates (14), including carp (15), exhibit precise diurnal rhythms with a peak during the dark phase and nadir during the photo phase and such rhythms are primarily generated by pineal AANAT (7). Until

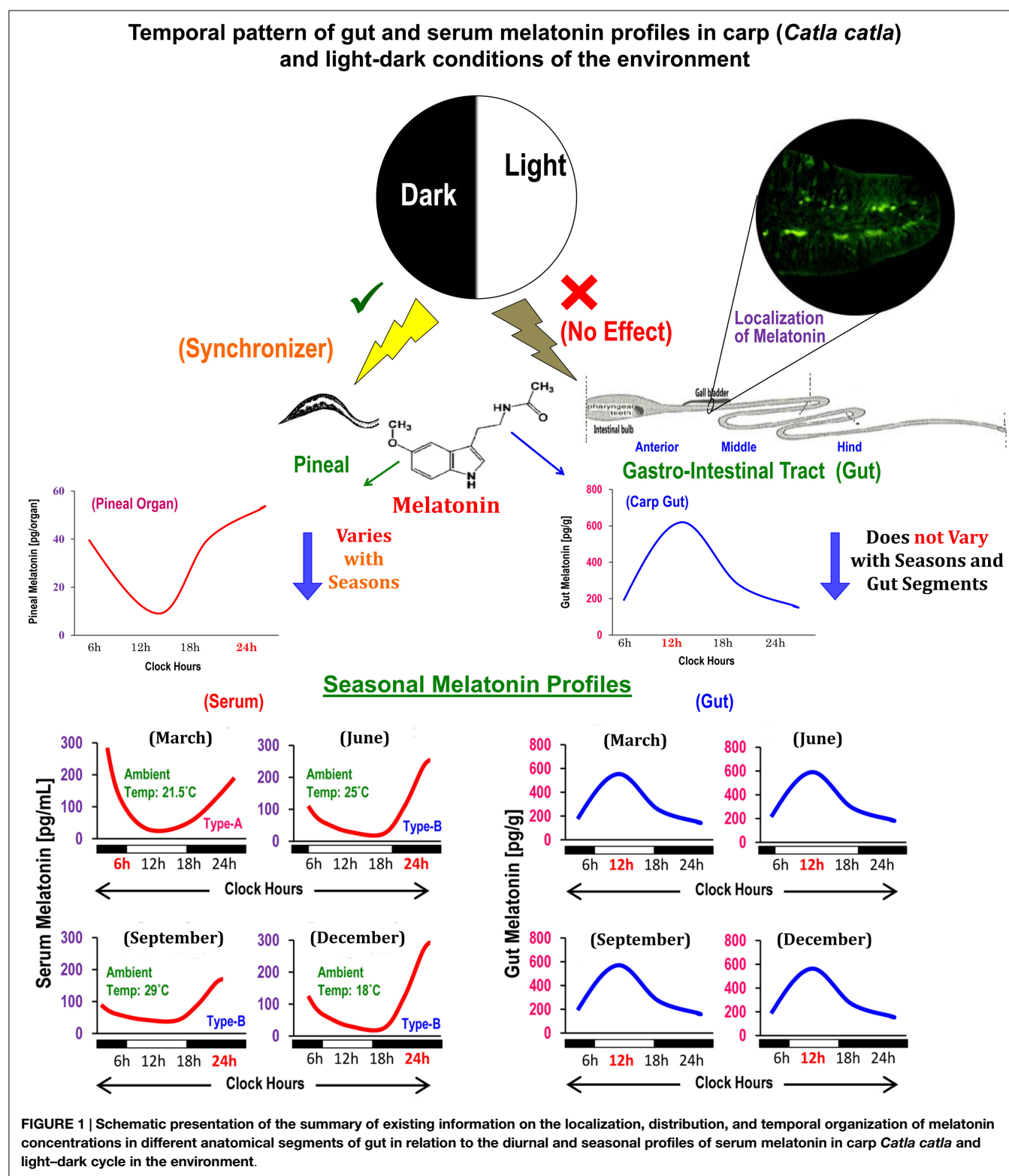
recently, comparable data on the temporal pattern of gut melatonin and its regulatory mechanisms were unknown for any vertebrates. A study on mice reported lower melatonin level during the day relative to nocturnal values only in the duodenum–jejunum segment of the GIT (16). In goldfish, though titers of melatonin were not measured, analysis of *Aanat*-2 mRNA expression revealed a daily rhythm in hindgut, but not in foregut (11). Importantly, the daily peak in the *Aanat*-2 mRNA expression persisted under continuous light as well as continuous darkness (11). Conversely, the study on carp by showing parallel changes in the levels of melatonin and AANAT density for the first time demonstrated that melatonin-synthesizing system in each gut segment, irrespective of seasons, undergoes significant daily variations with a peak at midday (6). Such findings also opened up a possibility that regulatory mechanism of melatonin synthesis in gut and pineal in the same animal species (Figure 1) is different (13).

Influence of Photoperiods on Rhythmicity in Gut Melatonin

The duration of light and thereby duration of darkness in a 24-h cycle is known to play a key role in the regulation of melatonin synthesis in the pineal (14). It is noteworthy that in fish, including carp (13), light acts directly on the pineal gland. By contrast, in mammals, light is detected by the retina and affects the pineal gland indirectly with a multisynaptic pathway (14). However, the question whether lighting conditions play a critical role of a synchronizer or regulator of gut melatonin was not duly addressed. Thus, a study was undertaken with carp (17), which due to its natural surface dwelling habit maintains close contact with environmental light and thereby is considered as an ideal model for the study of photo-response mechanisms in any melatonin-synthesizing tissue. Accordingly, the carp were held either under altered long (LP) or short photoperiods (SP) in a 24-h cycle, or under continuous light (LL) or darkness (DD) for equal duration. The results of the study indicated that none of the employed photo-regimens has any significant effect *per se* on the daily profiles of gut melatonin levels and AANAT protein density (17). Notably, an earlier study on the same carp demonstrated that diurnal rhythmicity in serum melatonin and pineal AANAT with a nocturnal peak (13) was lost when fish were held under LL or DD. Taken together, it is suggested that melatonin synthesis in gut, unlike pineal, is not a dark-dependent phenomenon, and environmental lighting conditions may not serve the role of external cue(s) in determining its rhythmic features in a daily cycle.

Influences of Food on Rhythmicity in Gut Melatonin

One of the periodic variables in the gut is the availability of food, which may serve as an important cue to determine daily periodicity of melatonin synthesis in gut (18). Notably, the study on the distribution of melatonin in different parts of the GIT in cow (poly-gastric) and pig (mono-gastric) revealed that cows had higher melatonin levels in stomach and ileum, but lower in cecum and colon (19). Accordingly, a relation between melatonin secretion and the digestive functions is sought. Depending on the meal frequency and timing of meals, several circulating metabolites and hormones undergo daily variations (20), and these variations



are dependent on whether the animal is fed, fasted, or starved. Because timing of food intake is roughly opposite in phase between diurnal and nocturnal species, it is likely that synthesis of gut melatonin is correlated with feeding in various animals

including fish species. However, any data from appropriate experimental studies that could support the hypothesis on a direct role of food availability and its timings on gut melatonergic system in any animal species still remain unknown.

Physiology of Gut Melatonin

Melatonin is a lipophilic compound diffusing rapidly through biological membranes and actions in an endocrine and/or, paracrine and/or, autocrine manners. It is involved in the regulation of multiple functions, including the control of gastrointestinal system. Melatonin produced by EC may perform paracrine functions, while its action in the intestinal muscles may be either direct or it may act via the myenteric nervous system (3, 19).

A study on rainbow trout demonstrated that melatonin is released from gastrointestinal tissue and addition of L-tryptophan to the incubation medium stimulates melatonin synthesis and release (21). The presence of melatonin in intestinal villi indicated that melatonin might be involved in the transmembrane transport of electrolytes and ions. Moreover, melatonin increased water content in feces (22) and the topical application of melatonin stimulated short-circuit current in colonic cells (23). Experimental studies confirmed that melatonin also inhibited the contraction of smooth muscles in stomach, ileum, and colon (24). In rats, endogenous melatonin affected the electro-myogram of pre- and post-prandial motility, though such actions were observed only at night (25), when the concentration of melatonin in blood remained usually high. Perhaps, melatonin relaxes gastrointestinal muscles by specifically blocking nicotinic channels (26).

The assumption that melatonin may also interact with dopamine-sensitive, possibly Ca^{2+} -activated, K^{+} channels (27) earned support from the study, in which inhibition of small-conductance K^{+} channels attenuated melatonin-induced relaxation of gastric muscles (28). In addition to its relaxation effect presented in several *in vitro* studies, melatonin appeared to inhibit serotonin (5-HT) action also *in vivo*. Serotonin facilitated the “food transit time” (FTT) (the moment of food intake to the appearance of the first feces stained with food coloring), as compared to controls, whereas injection of melatonin to serotonin implanted mice significantly increased the FTT (19). In a similar study, small doses of melatonin relaxed the gut muscles and facilitated intestinal motility in rats (19). Further studies argued that a counterbalancing system exists between serotonin and melatonin which within the GIT melatonin functions as a physiological antagonist of serotonin (22). High dose of melatonin inhibited the spontaneous or serotonin-induced contraction of GIT muscles and induced intestinal elongation. Conversely, low doses of melatonin stimulated intestinal contraction, resulting in the shortening of gut (3).

Several mammalian studies indicated that melatonin may have a protective role against development of gastric ulcers (29, 30). It is proposed that the prevention of stress- or ethanol-induced gastric lesions in rats is probably due to the anti-serotonergic effect of melatonin (29). The incidence and severity of spontaneously induced gastric ulcers are significantly reduced in pigs, which were

fed with 5 mg melatonin/kg enriched food. The highest incidence of ulcers is observed in pigs with the lowest level of melatonin in their plasma and stomach tissues (30). Protection against stress-induced lesions might be due to a strong antioxidant action of melatonin and the restoration of microcirculation (31).

In addition to its antioxidant effect, melatonin action in the prevention or treatment of colitis (32) includes the stimulation of the immune system. Though direct evidence is lacking, indications are available to show that melatonin administration to rats significantly increased the number and size of Peyer's patches, the major immune tissue of the GIT (33). Melatonin treatment may improve irritable bowel symptoms (IBS) as well (34). Thus, over the decades, melatonin has been promoted as a “magic cure” for the treatment or prevention of several physiological disorders ranging from aging to aggression, depression to hypertension, suppressed immunity to oxidative stress, insomnia to jet lag (1). However, convincing data are yet to be known to prove that such actions are also ascribed to gut-derived melatonin.

Conclusion

The information gathered so far provides indications that environmental and neuroendocrine regulatory mechanisms of melatonin synthesis in the pineal and in the gut are different. Likewise, functional characteristics of pineal-derived and GIT-derived melatonin may not be identical, as the nature of release and function of this extra-pineal melatonin are not yet fully understood. There are reasons to argue that pineal melatonin mostly acts as an endocrine agent, whereas GIT melatonin performs not only endocrine functions but also in autocrine and paracrine manners (18). However, emergence of a general idea on the physiological significance of gut melatonin has suffered a major setback due to lack of data from studies on any non-mammalian species and thereby warrants further carefully controlled research under diverse experimental conditions using animals representing different groups of vertebrates.

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Oligomerization of family B GPCRs: exploration in inter-family oligomer formation

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G-protein-coupled receptors (GPCRs) are classified into A to F subfamilies in which only families A, B, and C are present in mammals. Some of these GPCRs were found to form higher ordered structures such as oligomers with the discovery of interacting receptors in the form of homomers or heteromers. The importance of these oligomers on regulating receptor functions has recently been an intense research focus. It has been proposed that receptor oligomer formation has impact on its physiological importance on receptor trafficking, signaling, ligand-related regulation, and also is related to certain diseases. The present body of knowledge, however, comprises mainly intra-family oligomers formation and their consequences. Inter-family oligomers are recognized but there is limited information. This article aims to provide a current view regarding inter-family GPCR oligomerization in the subfamilies A, B, and C found in mammals.

Keywords: GPCR, family B, GPCR oligomerization, homomer, heteromer

INTRODUCTION

The vast structural and functional diversity of G-protein-coupled receptors (GPCRs) makes it the largest membrane receptor family. Members of the GPCR family include receptors responding to hormones, neurotransmitters, lipids, photons, ions, nucleotides, among others (1). It has been 20 years since GPCRs are classified according to the A to F system by Kolakowski in 1994. Under this system, only families A, B, and C are found in mammals (2). For these three families of receptors, rhodopsin-like receptors are classified as family A. Family B is further divided into three subfamilies by Harmar into subfamily B1 (secretin-like receptors), B2 (adhesion family), and B3 (Methuselah-like receptors) (3). Family C comprises members having characteristic long N- and C-termini and are responsible for sensing metabotropic glutamate, Ca^{2+} ion, and γ -aminobutyric acid (GABA) (4, 5). The concept of GPCRs oligomerization can be dated back to 1982 when Fraser and Venter discovered that β_2 -adrenergic receptors (β_2 -AR) form dimers in the cell membrane. Their studies provided the first piece of biophysical evidence of GPCR oligomerization using immunoaffinity chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and radiation inactivation techniques (6). Since then, receptor dimerization events were demonstrated by co-immunoprecipitation (co-ip) and resonance energy transfer approaches. Among the first studied cases of GPCRs oligomerization, most effort had been focused on family A GPCRs, while oligomerization in family B GPCRs was largely neglected until Miller's and Schelshorn's groups provided information on such events in the past decade. The Miller's group described the homo-oligomerization of secretin receptor (SCTR) (7, 8) and the heteromer formation of SCTR with other family B GPCRs (8, 9), while Schelshorn's group described glucagon (GCG), glucagon-like peptide-1 (GLP1), glucagon-like peptide-2 (GLP2), and gastric inhibitory polypeptide (GIP) receptors oligomerization (10–12). Apart from providing biophysical

proof of GPCRs interactions, physiological consequences of such interaction were also elucidated in the past 15 years when it was gradually unveiled that oligomerization of receptors plays roles in trafficking, ligand-promoted regulation, ligand binding, allosteric, as well as signal transduction (13). There also exist *in vivo* evidence that dimerization of family B SCTR with family A angiotensin II receptor type 1a (AT1aR) modulates the signaling properties of the receptors (14). In view of the emerging emphasis on inter-family GPCRs oligomerization and the functional importance of such event, this article will review the basis of GPCRs oligomerization emphasizing on family B GPCRs.

GPCR OLIGOMERIZATION

G-protein-coupled receptors were originally thought to function as monomeric molecules, with a 1:1 stoichiometry for one receptor protein to interact with one heterotrimeric G protein (13). However, with the advancement in receptor biology research, GPCR homo- and/or hetero-oligomerization is now generally accepted as a common phenomenon. Although the exact stoichiometry of such interaction remains mostly undetermined under the current limitation of technical difficulties, oligomers of GPCRs have proved to involve in vital roles in terms of functioning of the receptors.

For family A GPCRs, evidences have suggested dimerization as a prerequisite for correct trafficking as well as signaling of certain receptors. Examples are somatostatin receptor and adrenergic receptors (15, 16). Ligand may also regulate the oligomeric state of receptors on the cell surface, either positively for the luteinizing hormone receptor (17) or negatively for the thyrotropin receptor (18). Ligand binding cooperativity was observed to be altered in M2 muscarinic receptor dimers (19).

For family C receptors, receptor oligomerization is essential to the proper functioning of the protein. A classic example is the GABA_B receptor (GABA_BR), which is the heterodimer of the

protomers GABA_B1R and GABA_B2R. The ligand binding site is located on GABA_B1R but transportation to the cell membrane as well as G-protein coupling for proper functioning of the receptor can only be achieved when GABA_B2R is present and heterodimerizes with GABA_B1R (20, 21). The significance of family B receptors oligomerization will be discussed later in this article.

METHODS TO STUDY OLIGOMERIZATION OF GPCRS

Traditionally, co-ip was used to study receptor oligomers. Observations of oligomer formation in GPCRs primarily rest upon on this technique since the first report by Hebert et al. (22) describing β 2-AR receptor dimers (22). However, due to the technical difficulties, methods utilizing the resonance energy transfer between receptors tagged with luminescent or fluorescent proteins have become more popular. In one of the methods, known as bioluminescent resonance energy transfer (BRET), one of the receptor is tagged usually at the C-terminus with the enzyme *Renilla* luciferase (Rlu), which acts as the donor molecule, while the acceptor receptor is tagged with a yellow or green fluorescent protein (YFP/GFP). The two receptor constructs are co-expressed *in vitro*, usually in the form of a saturation experiment when a constant amount of the donor molecule is co-transfected with an increasing amount of acceptor receptors. Upon Rlu activation by its specific substrate, light energy from the reaction can be transferred and excite the acceptor fluorescent protein when the two receptors are in a proximity of <10 nm apart. This fluorescence of the acceptor emission can then be quantified as an indicator of receptor interactions known as the BRET signal. For specific interaction, the BRET signal will increase with increasing concentration of acceptor receptor, indicated by a saturation curve. Negative control is provided as a linear straight line when the BRET signal is produced by the random collision of tagged receptors. The advantages of using BRET methods over co-ip is that BRET improves resolution and more importantly, enable faster screening of dimerization partners (23, 24). Another similar technique replaces the Rlu with a cyan fluorescent protein (CFP), and requires the activation of CFP by laser for resonance energy transfer. This method is known as FRET and eliminates the use of substrate and is primarily useful in applications to study receptor trafficking under the microscope. However, it suffers the disadvantage of having to employ linear unmixing algorithms in the microscope software due to the possible crosstalk of fluorescent signals from the two fluorescent protein tags (25). An improvement to this situation relies on the use of long-lived rare-earth lanthanides energy donors, such as europium. This time-resolved FRET technique lowers the background signal and hence a higher signal:noise ratio can be obtained over normal FRET procedures (26). Other fluorescent based technologies include bimolecular fluorescence complementation and bimolecular luminescence complementation. These experiments use receptors tagged with a part of the fluorescent/luminescent protein that, upon receptor oligomerization, can reassemble into functional protein again (27). Although there exist different approaches to probe for receptor oligomers, most experimental evidence of the physiological consequences of these GPCRs interactions were only found in the *in vitro* model. *In vivo* evidence was poorly lacking until the demonstration of the phenotypes of luteinizing hormone receptor transgenic mice in

2010 (17), and water homeostasis effect of angiotensin II (ANGII) and SCTR homomers and heteromers in 2014 (14).

OLIGOMERIZATION OF FAMILY B GPCRS AND ITS FUNCTIONAL SIGNIFICANCE

Among the family B GPCRs, the SCTR is the first member of the family cloned from rat (28) and thus is the most comprehensively studied. Using primarily BRET technique, the Miller group provided evidences on SCTR homodimerization (8, 29, 30), as well as heterodimer formation with glucagon-like peptide-1 receptor (GLP1R), glucagon-like peptide-2 receptor (GLP2R), vasoactive intestinal peptide receptors 1 and 2 (VPAC1R, VPAC2R), growth hormone-releasing hormone receptor (GHRHR), parathyroid hormone 1 receptor (PTH1R), parathyroid hormone 2 receptor (PTH2R), and calcitonin receptor-like receptor (CRLR) (8, 9). The specific sites of interaction of SCTR homodimer were the transmembrane 4 of the receptor in which Gly243 and Ile247 residues play a key role (29).

For other members of the family, intra-family homomers includes GLP1R, GLP2R, gastric inhibitory polypeptide receptor (GIPR), glucagon receptor (GCGR) (10–12), PTHR1 (31), VPAC1R, VPAC2R (8), GHRHR (32), calcitonin receptor (CALCR) (33), CRLR (34), corticotrophin-releasing hormone receptor 1 (CRHR1) (35, 36), and pituitary adenylate cyclase-activating polypeptide type I receptor (PAC1) (37), while intra-family heteromers include VPAC1R/VPAC2R (8), GLP1R/GIPR, GLP1R/GCGR, GLP1R/GLP2R, GCGR/GIPR, GCGR/GLP2R, and GIPR/GLP2R (10–12).

For the family B receptors, physiological relevance of dimerization was mainly demonstrated by *in vitro* experiments. First being the dominant negative effect of a mis-spliced SCTR on wildtype SCTR, leading to gastrinoma development (7). The importance of dimerization for receptor trafficking is observed both in SCTR and VPAC1R in which non-dimerizing receptor constructs failed to reach the cell surface (8, 29, 30).

Although no effect was observed for SCTR homodimers (8), ligand binding can negatively affect dimer formation of VPAC1R/VPAC2R/PTH1R homo/heterodimers (8, 9, 31). Interestingly, GLP1 can positively affect dimer formation for GLP1R and GIPR dimer, while GIP has inhibitory effect on the hetero-complex formation (12, 38). In addition to ligand affecting dimer formation, formation of dimer has an effect on ligand binding as well. Using transmembrane domain 4 (TM4) peptide to disrupt GLP1R homodimer formation, binding of the ligand GLP1 (7–36)-NH₂ can be nullified (39). Truncated GHRHR can also lead to conformational change in the dimer complex, which is responsible for the inhibition of GHRH ligand binding (32).

Most family B GPCRs elicit their functions through the cAMP and/or phospholipase C (PLC) signaling cascade. The effect of oligomerization on the cellular mobilization of cAMP and calcium was also one of the research areas in GPCR oligomerization studies. In 2007, it was found that by disrupting SCTR homodimer formation using TM4 peptide, cAMP production is reduced (29). GLP1R and GIPR dimer is also known to decrease the maximal responses of GLP1R in terms of β -arrestin recruitment and calcium mobilization (38, 39). Taken together, oligomerization of GPCRs plays vitally important roles on multiple aspects of cell

physiology. **Table 1** outlines the intra-family oligomerization of family B GPCRs, the techniques involved and the physiological significance included.

INTER-FAMILY GPCRs OLIGOMERIZATION

Since the maturation of the concept of GPCR oligomerization, its functional implications are gradually understood and appreciated. Experimental evidences on oligomer formation as well as physiological importance has been accumulating first in families A and C, then in family B members in the past years. However, most studies so far has been focusing on intra-family receptor oligomerization, inter-family events are poorly understood. The fact that there exist little sequence homology between families A, B, and C receptors, despite having shared common general morphology, may explain this scarcity of information (5, 40). Up till now, documented inter-family events include family B GIPR with family A members β_2 -AR

and opsin. In their experiment, they use the BRET method in a heterologous expression system to discover that cAMP production is increased upon ligand stimulation compared to monomers (41). Family B CRHR1 with family A vasopressin receptor 1b (V1bR) was studied again using the BRET technique but instead of tagging the receptors at the C-termini, they are tagged at the N-termini (36). Family B GCGR with family A cholecystokinin A receptor was also reported (CCKAR) (10). Among these, family B SCTR with family A AT1aR shows the most convincing evidence. Based on the observation that both drinking behavior and vasopressin expression and release are impaired in secretin (SCT) or SCTR knockout mice upon intracerebroventricular (i.c.v.) injection of ANGII (42), it was deduced that a SCT/SCTR axis is essential to the proper functioning of ANGII in the central nervous system. With the coexpression of AT1aR and SCTR in the paraventricular nucleus, receptor heterocomplex formation was hypothesized to

Table 1 | Oligomerization of intra-family family B GPCRs.

| Oligomer | Technique | Physiological significance | Reference |
|-------------------|--------------------|---|--|
| HOMOMERS | | | |
| SCTR/SCTR | BRET/FRET | Relation with gastrinoma; promotes cAMP response | Ding et al. (7), Harikumar et al. (8, 29) |
| GLP1R/GLP1R | BRET | Promotes ligand binding | Orgaard (10), Roed (11), Schelshorn et al. (12), Harikumar et al. (39) |
| GLP2R/GLP2R | BRET | N/A | Schelshorn et al. (12) |
| GIPR/GIPR | BRET | N/A | Schelshorn et al. (12) |
| GCGR/GCGR | BRET | N/A | Orgaard (10), Roed (11), Schelshorn et al. (12) |
| PTHR1/PTHR1 | BRET/FRET | Ligand reduces oligomerization | Pioszak et al. (31) |
| VPAC1R/VPAC1R | Co-ip/BRET/FRET | Ligand reduces oligomerization | Harikumar et al. (8) |
| VPAC2R/VPAC2R | BRET/FRET | Ligand reduces oligomerization | Harikumar et al. (8) |
| GHRHR/GHRHR | Co-ip | Promotes ligand binding | McElvaine and Mayo (32) |
| CALCR/CALCR | Co-ip/BRET/FRET | Alters receptor trafficking | Seck et al. (33) |
| CRLR/CRLR | Co-ip/BRET/FRET | N/A | Heroux et al. (34) |
| CRHR1/CRHR1 | FRET/BRET | N/A | Kraetke et al. (35), Young et al. (36) |
| PAC1/PAC1 | Time-resolved FRET | N/A | Maurel et al. (37) |
| HETEROMERS | | | |
| SCTR/GLP1R | BRET | N/A | Harikumar et al. (9) |
| SCTR/GLP2R | BRET | N/A | Harikumar et al. (9) |
| SCTR/VPAC1R | FRET/BRET | Receptor trafficking | Harikumar et al. (8, 9, 29) |
| SCTR/VPAC2R | FRET/BRET | Receptor trafficking | Harikumar et al. (8, 9, 29) |
| SCTR/GHRHR | BRET | N/A | Harikumar et al. (9) |
| SCTR/PTHR1 | BRET | Ligands reduce oligomerization | Harikumar et al. (9) |
| SCTR/PTHR2 | BRET | Ligands reduce oligomerization | Harikumar et al. (9) |
| SCTR/CRLR | BRET | N/A | Harikumar et al. (9) |
| GCGR/GLP1R | BRET | N/A | Orgaard (10), Roed (11), Schelshorn et al. (12, 38) |
| GCGR/GLP2R | BRET | None | Schelshorn et al. (12, 38) |
| GCGR/GIPR | BRET | None | Schelshorn et al. (12, 38) |
| GIPR/GLP1R | BRET | GLP-1 induces oligomerization and flattened Ca^{2+} response; GIP reduces oligomerization but does not alter Ca^{2+} response | Harikumar et al. (39), Schelshorn et al. (12, 38) |
| GIPR/GLP2R | BRET | None | Schelshorn et al. (12, 38) |
| GLP1R/GLP2R | BRET | N/A | Orgaard (10), Roed (11), Schelshorn et al. (12, 38) |
| VPAC1R/VPAC2R | Co-ip/BRET | Ligand reduces oligomerization | Harikumar et al. (8) |

Technique involved and physiological significance are described; N/A, information not available.

be the reason behind this observation. By using BRET techniques, it was found that SCTR interacts with AT1aR specifically, but not angiotensin II receptor type 2 (AT2R). When the receptors were expressed together *in vitro* and stimulated with SCT alone, cAMP response was blunted compared to cells bearing only SCTR or SCTR with the non-dimerizing AT2R. However, cAMP production could be restored when ANGII was also present or when SCTR was co-expressed with a constitutively active mutant AT1aR, but not with the ANGII binding-deficient mutant AT1aR-K199A. Together with the fact that AT1aR cannot stimulate the cAMP signaling pathway, it was concluded that an active conformation of the AT1aR was responsible for regulating SCTR in mediating cAMP responses. In line with previous finding (29), the role of TM peptides on heterocomplex formation was also elucidated. It was found that peptides derived from SCTR's TM 2 and 4 (STM-II/IV), and AT1aR's TM 1 and 4 (ATM-1/4), could inhibit heteromer formation, while only STM-IV or/ATM-4 peptides could suppress the homomers of SCTR or AT1aR, with alanine mutants of these peptides reversing the situation. ATM-1 was chosen to investigate heteromer-specific actions as this peptide is neither capable of disrupting SCTR nor AT1aR homomer formation. By using these TM peptides as a tool, specifically ATM-1 and its mutant counterpart, it was demonstrated that full activity of SCT-stimulated SCTR requires the activation of AT1aR as a prerequisite. To further investigate the physiological relevance of SCTR/AT1aR heteromer in relation to water homeostasis, i.e.v. injections of the TM peptides and their mutants were administered to mice under hyperosmolality stress. All the four aforementioned TM peptides can suppress drinking behavior of these mice under hyperosmolality to different degrees. Of which, STM-IV, ATM-1, and ATM-4 can inhibit drinking behavior to a level similar to that of SCTR knockout mice or mice injected with the protein kinase A inhibitor H89, for the case of STM-II the effect was not as prominent. These results were reversed when the alanine mutants TM peptides were used instead. The experiments demonstrate that receptor heterocomplex of SCTR and AT1aR plays a role in regulating water drinking behavior *in vivo*. Furthermore, i.e.v. injection of a combination of low doses of both SCT and ANGII produces an effect on drinking behavior comparable to injection of high doses of these hormones alone, which is significantly greater than that when the hormones were given singly at low doses. This synergistic effect of the hormones in the central nervous system hints that the physiological effect of SCTR can be prominently enhanced with ANGII. This important study strongly suggests the significance of inter-family heterodimer formation on physiology (14). **Table 2** summarizes the heteromer formation of inter-family GPCRs.

CONCLUSION AND FUTURE PERSPECTIVE

Since there is a growing body of evidences on the functional importance of GPCR oligomerization but information on potential oligomerizing partners within families A, B, and C is lacking; the area of GPCRs oligomerization within these families is being explored in our laboratory recently. Initial approach includes setting up a GPCR–YFP library with GPCRs tagged with YFP. Using this scheme, any GPCR having tagged with Rlu can be screened against this library for oligomer partners. Not only can this study compensate our current paucity of information on inter-family

Table 2 | Oligomerization of inter-family family B GPCRs.

| Heteromers | Technique | Physiological significance | Reference |
|--------------------|-----------|--|-------------------|
| GIPR/ β 2-AR | BRET | Potentiates cAMP responses | Vrecl et al. (41) |
| GIPR/opsin | BRET | Potentiates cAMP responses | Vrecl et al. (41) |
| CRHR1/V1bR | BRET | N/A | Young et al. (36) |
| GCGR/CCKAR | BRET | N/A | Orgaard (10) |
| SCTR/AT1aR | BRET/FRET | Reduces secretin's cAMP response; alters drinking behavior | Lee et al. (14) |

Technique involved and physiological significance are described; N/A, information not available.

GPCR oligomers but also the cellular co-localization of these partners and their physiological relevance can be elucidated. If the receptors are found to be co-localized and serving similar functions, further biochemical analysis can be made to assay for the functional significance of the receptor interaction. As such, a number of novel mechanisms in controlling cellular activities may be discovered. Such discoveries could in turn facilitate the development of biochemical tools for scientific research or *in vitro* diagnostics. As around 30–40% of the pharmaceutical drugs at present are targeting GPCRs, this basic research on the role of GPCR oligomerization may pave the path for developing pharmaceutical precursors, which may eventually become the answers to a variety of diseases.

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Evolution of parathyroid hormone receptor family and their ligands in vertebrate

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The presence of the parathyroid hormones in vertebrates, including PTH, PTH-related peptide (PTHrP), and tuberoinfundibular peptide of 39 residues (TIP39), has been proposed to be the result of two rounds of whole genome duplication in the beginning of vertebrate diversification. Bioinformatics analyses, in particular chromosomal synteny study and the characterization of the PTH ligands and their receptors from various vertebrate species, provide evidence that strongly supports this hypothesis. In this mini-review, we summarize recent advances in studies regarding the molecular evolution and physiology of the PTH ligands and their receptors, with particular focus on non-mammalian vertebrates. In summary, the PTH family of peptides probably predates early vertebrate evolution, indicating a more ancient existence as well as a function of these peptides in invertebrates.

Keywords: parathyroid hormone, parathyroid hormone receptor, evolution, vertebrate, G protein-coupled receptor

Although the physiological actions of parathyroid hormone family are well-documented in the literature, review about the molecular evolution of PTH ligands and their receptors are limited. Therefore, in this article, we reviewed recent studies about the PTH ligands and their receptors in different vertebrate species. We believe this mini-review can provide a better overview about the molecular evolution of this ligand and receptor family in vertebrate.

PARATHYROID HORMONE FAMILY

The parathyroid hormone peptide subfamily includes PTH, PTH-related peptide (PTHrP), and tuberoinfundibular peptide of 39 residues (TIP39, also known as PTH2). These hormones are encoded in separate genes but their mature peptides share significant sequence homology within the first 34 amino acids. In humans, PTH is highly expressed and is secreted by the parathyroid gland, but lower levels of its transcript can also be detected in the hypothalamus, pituitary, and thymus (1–3). PTH is an important regulator for body calcium homeostasis. In contrast to PTH, PTHrP is widely expressed in a broad spectrum of tissues, including central nervous system (4). The main function of PTHrP is the regulation of chondrocyte growth and differentiation in the growth plates of developing long bones (5). TIP39 shares a relatively lower level of sequence homology with PTH or PTHrP, but high resolution NMR studies suggest that it has a similar three-dimensional structure when compared to them (6). TIP39 is mainly synthesized in two brain regions, the subparafascicular area of the thalamus and the medial paralemniscal nucleus of the pons (7). Recent reports suggest that TIP39 is a neuroendocrine hormone that modulates several aspects of the stress response, as well as controls body temperature (8). In addition to PTH, PTHrP, and TIP39, there is a newly identified member of the family, PTH-like peptide (PTH-L), which is only present in non-mammalian species including *Xenopus*, teleosts, and chicken. *pth-l* gene structure is similar to that of the *pth* gene, but the mature peptide of PTH-L

shares a higher level of sequence homology to PTHrP (9). The expression of PTH-L has been investigated in chicken and *Xenopus*. The peptide was found widely, but differentially expressed in various tissues of these organisms. Abundant PTH-L transcripts were detected in cartilage in chicken, and in brain, lung, and bone in *Xenopus laevis* (10). Although the physiological role has not yet been fully established, some reports have suggested that PTH-L in teleosts (Seabream) and *X. laevis* is the most potent calciotropic factor among all PTH peptides (10, 11).

PTH RECEPTOR FAMILY

The importance of the PTH system as a regulator to control blood calcium levels was recognized early in the 20th century by observing the correlation between the parathyroid gland and tetany (12, 13). However, understanding of the physiological function of PTH was not substantially developed until the discovery of the human PTH1R in 1991 (14). PTH1R is also called the PTH/PTHrP receptor because of its equal binding affinities for both PTH and PTHrP. Subsequently, PTH2R, with a ligand preference for PTH over PTHrP, was identified in humans (15). However, further investigations suggested that TIP39 is the native ligand of PTH2R because of the robust activation of human PTH2R by TIP39 and the poor activation of rat PTH2R by PTH (16). In the last two decades, due to the success of different genome projects, paralogs and orthologs of PTHRs have been identified from various vertebrate species. These studies have also led to the discovery of an additional member of the receptor family, PTH3R, which is found only in non-mammalian vertebrates. The ligand specificity and structure of PTH3R resemble PTH1R more than PTH2R (17–19). In zebrafish, seabream, and chicken, PTH3R shows a stronger affinity for PTHrP than do other members of the PTH receptor family (17, 18, 20).

EVOLUTION OF PTH AND THE PTHR FAMILY

The PTHRs belong to the class B1 G protein-coupled receptors (GPCRs). It is widely believed that the paralogs of PTHR, *pth1r*,

pth2r, and *pth3r* are evolved after two rounds of whole genome duplication (2WGD) (17, 21–23). Recent genome synteny comparison provided strong evidence to support this hypothesis (22). From teleosts to humans, PTHRs are found in three conserved loci. Interestingly, PTH2R and PTH3R are not found in birds and mammals, respectively. The genes for the PTHR cognate ligands (*pth*, *pthrp*, and *tip39*) are also located in three conserved loci and were likely generated through similar WGD events as their receptors. The *pth-l* gene is found in the fourth locus in chicken, which shares considerable synteny with the other three conserved loci of the *pth* gene family. Specifically in teleost, the third WGD generate extra copies of *pth* (*pth1* and *pth2*) and *pthrp* (*pthrpA* and *pthrpB*), but whether the copies share the synteny with PTH gene family remains to be investigated (11, 24).

The elephant shark was diverged at the time point just after the vertebrate 2WGD; therefore, its primitive genome provides insight into the evolution of the PTH and PTHR family (25, 26). Consistent with the “4:1 rule” (27, 28), except for the loss of the fourth putative PTHR, all the homologs of PTH and the PTHR genes could be identified in elephant shark (hereafter abbreviated to “e”) (17, 29). PTH, PTHrP, and putative ePTH-L (designated as PTH2 in the references) have already been cloned, while a putative *tip39* gene was identified in the genome database (17, 29). Even though ePTH2 is phylogenetically grouped with other PTH-Ls, the identity of ePTH2 as an ortholog to fish PTH-L was questioned because the gene structure of *ePTH2* is different from teleost *pth-l*, but resembles other *pth* genes. Elephant shark *pth2* has its first intron before the KR cleavage site; whereas teleost *pth-l* genes do not contain this cleavage site, and instead have the first intron before the SRR motif (29). More importantly, ePTH2 is unable to stimulate cAMP production in PTH1R expressing cells, but teleost PTH-L appears to be a potent factor (29).

Regarding the evolution of PTH and the PTHR family in early vertebrates, hypotheses have been proposed largely based on bioinformatics data (17, 21, 22, 30). In lamprey, the distinct phylogenetic positions of the two deduced PTH-like receptors and hormones, suggested a possible scenario of PTHR/PTH evolution before 2WGD (17). PTH1R-like and PTH2R-like genes were produced after the first round of WGD. Then, after the divergence of lamprey, the second round of WGD resulted in only PTH3R due to the lost of the fourth receptor in the PTH2R lineage. For the ligands, PTHrP and TIP39 were proposed to be the pioneers of the PTH family, since they are present in agnathan genomes (18).

In invertebrates, PTHR-like genes are found in the genomes of protostomians, cephalochordates, and urochordates. This indicates the ancestral PTHR was evolved before the deuterostome-protostome split (21, 22, 30). Regarding the ligands, a recent bioinformatics approach has identified PTH-like peptides in tunicates and amphioxys (31). Even though the invertebrate peptides share relatively low homology to the vertebrate PTH family, these observations indicate that the PTH family of peptides already existed in cephalochordates.

EVOLUTIONARY CHANGES IN GENE STRUCTURES OF THE PTH FAMILY

After divergence from their last common ancestor, PTHrP exhibited more changes in gene structure than PTH. The classic gene

structure of *pth* contains three exons with the prepro-peptide are encoded in the last two exons. This gene structure is conserved from elephant shark to human (10, 24, 29). However, the *pthrp* gene structure differs through vertebrates by the introduction of exons upstream and/or downstream of the mature peptide coding regions, and presence of splicing variants in *Xenopus*, chicken, and human (10, 32). In addition, unlike PTH and PTH-L, their precursor proteins that lead to single mature peptide, posttranslational processing of mammalian prepro-PTHrP can give rise to three mature peptides: PTHrP, middle region, and osteostatin (33, 34). The increase of *pthrp* gene structure complexity during vertebrate evolution may reflect changes in the physiological roles of this peptide, such as adaptation to the terrestrial environment in tetrapods (9, 35). The new member of the PTH family, PTH-L in non-mammalian vertebrates, was considered as an intermediate between PTH and PTHrP due to its independent phylogenetic position to both PTH and PTHrP (10, 11). Moreover, although the gene structure of PTH-L resembles that of PTH in fugu, chicken, and *Xenopus*, alternative transcripts were reported for *Xenopus* and chicken PTH-L. In addition, all investigated PTH-L peptides contain the “MHD” motif, which is the characteristic of teleost PTHrP (9–11). In elephant shark, all the PTH peptides possess the “MHD” motif (29), suggesting that this motif is present in the common ancestor of PTH peptides and was lost during evolution in some vertebrates. Among all PTH family members, the unique properties of TIP39 are shown by its distant position from other members in phylogenetic analysis, and by the lack of “MHD” or any similar motif (36, 37). The gene structure of TIP39 is highly conserved from mammals to teleosts, and like that of PTH, has three exons (37). The uniqueness of TIP39 could be a result of its earlier divergence from other members of the PTH family.

LIGAND BINDING OF PTHRs IN VERTEBRATES

Significant levels of sequence homology are found in the first 34 amino acids of mature PTHrP, PTH, and PTH-L peptides. This region is important for receptor binding and receptor activation (9, 10, 16). In mammals, it has been shown that activation of PTH1R by PTH_(1–34) and PTHrP_(1–34) are comparable to that by the corresponding full length peptides (38, 39). The biological activity of PTH_(1–34) was indicated by administration of PTH_(1–34) to patients with osteoporosis, which resulted in enhanced bone development (40, 41). PTH_(15–34) are required for high affinity binding through interaction with the extracellular N-terminal domain of the receptor (42, 43). Such functional division within PTH in receptor interaction is also likely for PTHrP and TIP39, as well as for interaction of PTH2R with PTH-like peptides, as shown by studies of ligand variants and chimeric receptors (44–47). Functional testing using truncated PTHrP_(2–34, 3–34, and 7–34) indicated that these peptides were unable to trigger cAMP synthesis but could stimulate the PLC pathway. In summary, the first few residues are essential to the cAMP pathway but not for receptor binding (48). These properties of truncated PTH_(7–34) and PTHrP_(7–36) were utilized for developing antagonists of PTH1R (47).

Table 1 summarizes the ligand specificity of the PTHR family in various species. Human (hu) PTH1R can be activated by PTH and PTHrP with indistinguishable potency in downstream

Table 1 | Ligand specificity of PTHR family.

| Receptor | Species | Assay | Ligand specificity | Reference |
|----------|-----------------------|----------------------|---|-----------|
| PTH1R | Human | cAMP | hPTHrP \approx hPTH \approx zPTH1 \approx zPTH2 > fPTHrP, no response to TIP39 | (14, 24) |
| | | PLC | hPTHrP \approx hPTH | (19) |
| | | ERK1/2 | hPTHrP \approx hPTH | (51) |
| | | IP1 | hPTHrP \approx hPTH | (51) |
| | | Binding ^c | hPTHrP \approx hPTH | (52) |
| | Mouse | cAMP | hPTHrP \approx hPTH, no response to TIP39 | (50) |
| | Rat | cAMP | rPTHrP \approx rPTH, no response to TIP39 | (16) |
| | Chicken | cAMP | cPTHrP \approx hPTH > cPTH-L > cPTH | (17) |
| | | PLC | cPTHrP, slight stimulation by cPTH-L and no response to and cPTH | (17) |
| | Zebrafish | cAMP | hPTH \approx hPTHrP \approx fPTHrPA \approx zPTH1 > zPTH2 | (18, 24) |
| PTH2R | Human | PLC | hPTHrP \approx hPTH | (18) |
| | | cAMP | fPTH-L \approx fPTHrPA > hPTH \approx bPTH, low respond to hPTHrP, no response to fPTH, and fPTHrPb | (11) |
| | | PLC | fPTHrPA \approx hPTHrP \approx hPTH \approx fPTH-L \approx fPTHa \approx fPTHb, no response to fPTHrPB and bPTH | (11) |
| | | cAMP | hTIP39 \approx rTIP39 \approx rPTH > hPTH | (53, 54) |
| | | PLC | hTIP39 \approx rTIP39 > rPTH, no response to hPTH | (53) |
| | Rat | cAMP | hTIP39 > rPTH | (16) |
| | Zebrafish | cAMP | hTIP39 \approx zTIP39 > hPTH | (36) |
| | (SV) | cAMP | zTIP39 > hTIP39 | (36) |
| | Chicken | cAMP | cPTHr > cPTH, low response to cPTH-L | (17) |
| | Zebrafish | cAMP | hPTHrP \approx fPTHrPA \approx zPTH1 > zPTH2 > hPTH | (18, 24) |
| PTH3R | Chicken | PLC | no response to hPTHrP and hPTH | (18) |
| | | cAMP | fPTHrP, no response to hPTHrP and hPTH | (20) |
| | Seabream ^b | cAMP | fPTHrP, no response to hPTHrP and hPTH | (20) |
| | | PLC | no response to fPTHrP | (20) |

^a Scale.^b Enterocyte.^c Extra-cellular domain.SV, splice variant; h, human; r, rat; z, zebrafish; f, fugu; c, chicken; x, *Xenopus*.

signaling pathways, including cAMP, phospholipase C (PLC), and ERK1/2 (49). No activity has been detected for TIP39 in any mammalian PTH1R. It is interesting to note that zebrafish (zf) PTH is able to activate human PTH1R with similar potency to huPTH (24). This indicates the overall structures of the peptides and the receptor binding pockets are highly conserved from mammals to teleosts. Similar ligand specificity is observed in rodents (16, 50) and teleosts (18, 24). In teleosts, fugu (fu) PTHrPA, huPTHrP, and zfPTH exhibit similar efficacies for cAMP activation in zfPTH1R. The only exceptional case is chicken: cPTH1R can be activated by chicken (c) PTHrP as well as by huPTH with similar potency, but to a lower extent by cPTH and cPTH-L (17, 18, 24).

Regarding PTH2R, TIP39 was shown to be the native ligand of PTH2R in mammals and zebrafish (16, 36, 53, 54). huPTH2R responded similarly to TIP39 and PTH in cAMP activation, but PTHs were less effective in PLC activation (53). As was the case for PTH1R, cross-species ligand reactivity is found in zfPTH2R; huTIP39 has similar potency to the endogenous ligand. In contrast, activation of the PLC has not been reported in PTH3R studies. Treatment of zfPTH3R with huPTHrP or PTH did not result in PLC stimulation, and consistent results were observed on treatment of seabream PTH3R with fuPTHrP (18, 20). The ligand and bias of PTH3R varies in different species. zfPTH3R shows a

preference for fuPTHrP over huPTH, whereas chPTH3R responds similarly to chicken PTHrP and PTH (17, 18). For seabream PTH3R, only fuPTHrPA could activate the receptor (20). In summary, characterization of the PTHR family in various species shows that their ligand specificity is well conserved. Cross-species ligand reactivity of PTHRs demonstrates that PTHRs share similar ligand-receptor binding properties that are conserved throughout vertebrate evolution.

FUNCTIONAL CHANGES IN THE PTH-PTHR SYSTEM DURING VERTEBRATE EVOLUTION

Due to the discovery of the relieving effect of PTH and PTHrP on tetany resulted from parathyroidectomy (12), and also that PTH and PTHrP are related to humoral hypercalcemic syndrome (55–57), initial investigations described the PTH peptides as endocrine hypercalcemic factors. Later research on PTHrP and TIP39 explored other possible physiological functions of these peptides. The vital role of PTHrP in fetal bone development was demonstrated in mouse models by deletion of the PTHrP gene and knockout of PTH1R (58–60). Much work has been undertaken to determine the wider physiological importance of PTHrP and has indicated that PTHrP is a multifunctional paracrine/autocrine factor (61). So far, in placental mammals,

accumulated knowledge of the widespread PTH/PTHrP-PTH1R system implicates its pleiotropic functions in regulation of calcium levels including: (1) the control of the release of calcium from bone and increase in renal calcium reabsorption; (2) its effect in the development of bone, cartilage (62–64), pancreas (65, 66), tooth (67–70), and mammary gland (62); and (3) its function to regulate placental calcium supply to the fetus (63, 64, 71). In addition, the C-terminal regions of PTH and PTHrP were postulated to interact with other yet-to-identify receptors, with potential functions remain to be explored (72–74). On the other hand, the expression of PTH2R is restricted to the central nervous system, and the TIP39-PTH2R system is involved in nociceptive signal processing, regulation of hormonal release from the hypothalamus-pituitary axis, and modulation of affective behaviors (44). The distinct and diverse functions of these peptides observed in mammals raised the question of what are the ancestral functions of the PTH-like system in non-mammalian vertebrates and even in invertebrates. Unfortunately, the physiological functions of most PTH peptides in non-mammalian species have not been investigated in depth, and functions of these peptides are largely proposed based on the spatial distribution of their proteins and mRNAs.

The evolution of the parathyroid gland was a key event in the emergence of the tetrapods. Therefore, the expression of PTH in the parathyroid gland was originally linked to an evolutionary concept that the emergence of the PTH-PTHrP system was co-evolved with the adaptive transition from calcium-rich aquatic to calcium-deficient terrestrial habitats (9, 75). However, the unexpected identification of PTH in teleosts completely changed this view. Now, the cloning of two PTHs from elephant shark confirms that PTH-like peptides were present far back in evolution as cartilaginous fish, suggesting the original role of these peptides is unrelated to bone formation (29). The endocrine action of PTH released from parathyroid glands in mammals could not be observed in fish, which have no parathyroid gland. Instead, it was suggested that PTH is a paracrine factor in non-mammalian vertebrates such as fish and *Xenopus* (10). Although the function of PTH in fish remains poorly described, PTH-L which is absent from placental mammals, was proposed to mimic the role of mammalian PTH in fish, since fuPTH-L was found to be a potent factor causing whole body calcium influx in seabream larvae but no response was detected using fugu PTHA or PTHB (11). Based on the recent characterization of PTH-L from chicken and *Xenopus*, it was hypothesized that there was a functional transition between PTH-L and PTH during vertebrate evolution. In this scenario, PTH-L gradually lost its calcitropic activity and eventually was lost in mammalian genomes, while PTH replaced PTH-L in mammals to become the main endocrine regulator of calcium with expression restricted only to the parathyroid gland (10). One piece of supporting evidence for such a transition is that PTH-L and PTHrP in chicken and *Xenopus* show overlapping tissue distributions, indicating redundancy. Similarly, immunohistochemistry in elephant shark showed that the PTH orthologs ,PTH1 and PTH2, exhibit widespread localization and considerable locational overlap with PTHrP (29). This implies that PTH-like peptides in non-mammalian vertebrates and cartilaginous fish may partially share their physiological role(s) with PTHrP. However, there is a mismatch between the results of widespread protein detection and

restricted mRNA expression of elephant shark PTH1 and PTH2 (29). Whether this reflects the endocrine action of the two peptides remains to be determined.

Phylogenetic analysis of two predicted PTH-like in the lamprey genome, TIP39 and PTHrP, suggests that these are probably the ancestral members of the PTH peptide family found in vertebrates (17). In general, the expression of these two peptides appears to be unchanged in the vertebrate lineage (76). Unfortunately, no experiments have been conducted to determine expression patterns of lamprey TIP39. Wide tissue expression of PTHrP was observed in all investigated species to date, and its detection in skin, skeletal and, cardiac muscle, and kidney is conserved from lamprey to human (9, 29, 77, 78). The very similar tissue expression patterns of TIP39 and PTHrP in zebrafish and lamprey respectively indicate that TIP39 and PTHrP possess ancestral functions compared with PTH and PTH-L. In invertebrates, use of heterologous antisera in immunohistochemistry enabled detection of PTH-like peptides in snail, cockroach, and amphioxus neural tissue (79). This indicates that the origin of the PTH family may be far earlier in evolution than our expectation.

CONCLUSION

Based on bioinformatics, the presence of PTHR dates back to an ancestor before the deuterostome-protostome split. Although the true identity of PTH-like peptides in invertebrates requires clarification, the peptide family likely co-evolved with its cognate receptors in vertebrates since agnatha. Duplication of this ancestral PTHR through 2WGD resulted in the PTHR family found in modern jawed vertebrate species. Insight from the recent characterization of PTH and the PTHR family from non-mammalian species, and the discovery of putative PTHR-like and PTH-like, in a lamprey genome, reveals that the pioneer of the PTH-PTHrP system, and its physiological properties, are likely fundamentally conserved throughout vertebrate evolution.

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Identification, functional characterization, and pharmacological profile of a serotonin type-2b receptor in the medically important insect, *Rhodnius prolixus*

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In the Chagas disease vector, *Rhodnius prolixus*, two diuretic hormones act synergistically to dramatically increase fluid secretion by the Malpighian tubules (MTs) during the rapid diuresis that is initiated upon engorgement of vertebrate blood. One of these diuretic hormones is the biogenic amine, serotonin (5-hydroxytryptamine, 5-HT), which controls a variety of additional activities including cuticle plasticization, salivary gland secretion, anterior midgut absorption, cardioacceleratory activity, and myotropic activities on a number of visceral tissues. To better understand the regulatory mechanisms linked to these various physiological actions of serotonin, we have isolated and characterized a serotonin type 2b receptor in *R. prolixus*, Rhopr5HTR2b, which shares sequence similarity to the vertebrate serotonin type 2 receptors. *Rhopr5HTR2b* transcript is enriched in well-recognized physiological targets of serotonin, including the MTs, salivary glands and dorsal vessel (i.e., insect heart). Notably, *Rhopr5HTR2b* was not enriched in the anterior midgut where serotonin stimulates absorption and elicits myotropic control. Using a heterologous functional receptor assay, we examined Rhopr5HTR2b activation characteristics and its sensitivity to potential agonists, antagonists, and other biogenic amines. Rhopr5HTR2b is dose-dependently activated by serotonin with an EC₅₀ in the nanomolar range. Rhopr5HTR2b is sensitive to alpha-methyl serotonin and is inhibited by a variety of serotonin receptor antagonists, including propranolol, spiperone, ketanserin, mianserin, and cyproheptadine. In contrast, the cardioacceleratory activity of serotonin revealed a unique pharmacological profile, with no significant response induced by alpha-methyl serotonin and insensitivity to ketanserin and mianserin. This distinct agonist/antagonist profile indicates that a separate serotonin receptor type may mediate cardiomodulatory effects controlled by serotonin in *R. prolixus*.

Keywords: serotonin, 5-hydroxytryptamine (5-HT), G protein-coupled receptor (GPCR), diuresis, dorsal vessel, Malpighian tubules, serotonergic signaling, cardioacceleratory activity

Introduction

The widespread presence and diverse biological roles of the indoleamine, serotonin (5-hydroxytryptamine, 5-HT), spans protozoans, plants and the vast majority of metazoans, thus attesting to its evolutionary significance (see Turlejski, 1996). In mammals, and more specifically in humans, serotonin acts on many organ systems via 7 families of serotonin receptors (5-HT_{1–7}) that constitute numerous receptor isoforms (see Pytliak et al., 2011; Verlinden et al., 2015). The serotonin receptor, 5-HT₃, is a ligand-gated channel whereas all other serotonin receptor classes have been characterized as G-protein coupled receptors (GPCRs) (Millan et al., 2008). Released as both a neurotransmitter and a hormone, the functions of serotonin are extensive, ranging from regulating the mechanics of behaviors like learning, memory, perception, fear and appetite, to mediating a plethora of physiological processes (see Berger et al., 2009; Verlinden et al., 2015).

The similarity in serotonin's regulatory functions in vertebrates as well as invertebrates is noteworthy. To name a few, serotonin is associated with learning and memory in sea slugs (Rahn et al., 2013), social and anxiety-like behavior in crayfish (Momohara et al., 2013; Fossat et al., 2014) and aversive behavior in the nematode, *Caenorhabditis elegans* (Curran and Chalasani, 2012). Serotonin is also a key modulator of feeding-related behaviors, including salivation, bite-like movements, pharyngeal peristalsis, and control of blood meal ingestion in a blood-feeding aquatic invertebrate, the medicinal leech, *Hirudo medicinalis* (Lent and Dickinson, 1987, 1988; Lent et al., 1988).

In insects, serotonin influences feeding-associated behaviors in various species including the locust (Ali et al., 1993; Molaei and Lange, 2003), blowfly (Baumann and Walz, 2012), honeybee (French et al., 2014), cockroach (Troppmann et al., 2007), as well as an ant (Falibene et al., 2012). Studies in *Drosophila melanogaster* and *Apis mellifera* have linked serotonin to development and various behaviors associated with central pattern generators, such as olfaction, learning, memory and circadian rhythms (Blenau and Thamm, 2011; Johnson et al., 2011). The immunohistochemical mapping of serotonergic neurons in the nervous systems of several dipteran, orthopteran, lepidopteran, blattarian, and hemipteran species further emphasizes serotonin's role as a neurotransmitter and neurohormone in insects (Nassel, 1988; Bicker, 1999; Miggiani et al., 1999; Homberg, 2002; Siju et al., 2008).

Rhodnius prolixus, a chief vector of Chagas disease, depends on its serotonergic system to successfully complete blood meal engorgement and, even more importantly, to regulate haemolymph osmolarity after its dramatic feeding bout. On average, a fifth instar *R. prolixus* can consume blood meals that are 10 times its unfed body weight and then promptly eliminates excess water and ions via rapid hormone-controlled diuresis (Orchard, 2006, 2009). Serotonin is a principal diuretic hormone in *R. prolixus* and haemolymph titres of serotonin rise dramatically from low nanomolar (<10 nM) to high nanomolar (>100 nM) levels within 5 min of feeding (Lange et al., 1989; Maddrell et al., 1991). Upon its release via

serotonergic neurohaemal sites, serotonin stimulates diuresis, muscle contractions of the dorsal vessel, salivary glands, esophagus (foregut), anterior midgut (i.e., crop), and hindgut (Orchard and Te Brugge, 2002; Orchard, 2006). In addition, serotonin is involved with the plasticization of the cuticle and the expulsion of waste (Orchard et al., 1988; Lange et al., 1989).

Along with a corticotropin-releasing factor (CRF)-like peptide, RhoprCRF/DH, serotonin is involved in the production of primary urine by stimulating secretion of excess water and ions by the Malpighian tubules (MTs) (see Martini et al., 2007). Unlike most other tissues where serotonin is known to play a role, the MTs lack innervation and are thus influenced by the rise in the haemolymph levels of serotonin, acting as a neurohormone (Lange et al., 1989). The presence of serotonin receptors on the epithelial cells of the MTs is therefore critical to the normal course of rapid post-feeding diuresis in *R. prolixus*. Interestingly, serotonin and RhoprCRF/DH have been shown to synergistically increase fluid secretion in the MTs, and both appear to be at least partially dependent on both intracellular calcium and cAMP-mediated signal transduction pathways (Paluzzi et al., 2013). A recent study corroborating the requirement of both intracellular calcium and cAMP revealed that serotonin triggers calcium signaling (derived specifically from intracellular stores), which is mediated by cAMP and cAMP-dependent kinase, protein kinase A (Gioino et al., 2014).

Based on similarity to the mammalian serotonin receptor families, research in insects has identified six serotonin receptor subtypes, including type 1a, type 1b, type 2a, type 2b, type 7, (Tierney, 2001; Vleugels et al., 2013, 2014; Verlinden et al., 2015) and a novel type 8, recently identified in the small white butterfly, *Pieris rapae* (Qi et al., 2014). In comparison to the vertebrate receptors, the insect serotonin receptors have different pharmacology and may utilize varying modes of signal transduction (Vleugels et al., 2014; Verlinden et al., 2015). Serotonin receptors have been predicted and/or cloned from several insects and their endogenous roles are now being explored (Von Nickisch-Rosenegk et al., 1996; Pietrantonio et al., 2001; Dacks et al., 2006b; Hauser et al., 2006, 2008; Troppmann et al., 2010; Gasque et al., 2013; Thamm et al., 2013; Vleugels et al., 2013, 2014).

In spite of the wealth of knowledge on serotonin's physiological roles in *R. prolixus*, surprisingly little is known concerning their endogenous serotonin receptors. In addition, we were interested to see if there might be more than one serotonin receptor in the periphery mediating the physiological effects of serotonin. We chose a tissue that is not directly involved in the rapid post-feeding diuresis. Thus, we examined the effects of serotonin agonists and antagonists on serotonin-stimulated increases in heart beat frequency (see Orchard, 2006). The susceptibility of these serotonin receptors to agonists and antagonists could then be compared to the pharmacology of the cloned receptor. The overall aim of this study was to address this knowledge gap and to begin to identify the serotonin receptors that mediate the various functional activities of serotonin in *R. prolixus*.

Materials and Methods

Animals

Fifth instar *R. prolixus* were obtained from an established colony at the University of Toronto Mississauga. Insects were reared in incubators at 25°C under high humidity (~50%). Each post-embryonic developmental stage was blood fed through an artificial feeding membrane as described previously (Paluzzi et al., 2015) using defibrinated rabbit blood purchased from a local supplier (Cedarlane Laboratories Inc., Burlington, ON). During dissection of animals to retrieve RNA from different tissues, the insects were bathed in nuclease-free phosphate-buffered saline (PBS) (Sigma-Aldrich, Oakville, ON, Canada) and excised tissues were transferred directly into chilled RNA lysis buffer (see below).

Isolation of a *R. prolixus* Putative Serotonin Receptor cDNA

Based on the pharmacological sensitivity of serotonin-stimulated fluid secretion by isolated *R. prolixus* MTs to ketanserin (Maddrell et al., 1991; Te Brugge et al., 2001), a selective antagonist of serotonin type-2 receptors (Hedner and Persson, 1988), the *D. melanogaster* type-2A serotonin receptor (Colas et al., 1995) protein sequence (Genbank accession# CAA57429) was used in a local tblastn search of the *R. prolixus* preliminary

genome using Geneious 6.1 software (Biomatters Ltd. Auckland, New Zealand) and genomic regions with high scoring matches were used to design several pairs of gene-specific primers (Sigma Aldrich, Oakville, ON, Canada). Using a previously prepared *R. prolixus* Malpighian tubule cDNA library (Paluzzi et al., 2010) as template, only one of these primer pairs (5htR2-forA and 5htR2-revA) was successful (see Table 1; unsuccessful primer pairs are not shown), which amplified an initial 263 bp cDNA product. PCR reactions used ThermoPol Taq Polymerase (New England Biolabs, Whitby, ON, Canada) following manufacturer recommendations and carried out on a PCR System 9700 thermal cycler (Perkin Elmer Applied Biosystems, Carlsbad, CA, USA) using the following settings: initial denaturation for 5 min at 95°C; 35 cycles of 1) denaturation for 1 min at 94°C, 2) annealing for 30 s at 58°C, and 3) extension for 30 s at 72°C; final extension for 10 min at 72°C. The amplified cDNA sequence was cloned into a sequencing vector, pGEM T-Easy (Promega Corporation, Madison, WI, USA) and bases identified by Sanger sequencing (Center for Applied Genomics, Hospital for Sick Children, Toronto, ON). The putative translation of this preliminary product was compared to the UniProt protein database using the Expasy Bioinformatics Resource Portal (<http://web.expasy.org/blast/>) and found to be highly similar to several uncharacterized protein sequences including a *D. melanogaster* orphan receptor (Genbank accession#: AGP51353.1) having

TABLE 1 | Primer sequences used in this study.

| Oligo name | Oligo sequence (5'-3') | Description (cDNA nucleotide binding site) |
|-----------------|-----------------------------|---|
| 5htR2-forA | TCGCGCACTTCATCTCG | Cloning initial partial cDNA (1969-1985) |
| 5htR2-revA | TTCTTGAATGCTTGTGCAAAAC | Cloning initial partial cDNA (2231-2211) |
| 5htR2-for1 | GCAGCTGGCAACATCC | Further cloning partial cDNA (250-265) |
| 5htR2-for2 | AGTTTACTGTTTGGCGTGG | Further cloning partial cDNA (417-435) |
| 5htR2-for3 | CAAATACTATAGTAACATGGGATTCC | Further cloning partial cDNA (1688-1713) |
| 5htR2-rev1 | CGATATCGACACAATAAGACTTTTC | Further cloning partial cDNA (2252-2229) |
| 5htR2-rev2 | CTAAGTGGAAGACTCATAGCTATCG | Further cloning partial cDNA (614-590) |
| 5htR2-rev3 | GCATGACGAGTATGGCGAC | Further cloning partial cDNA (370-352) |
| 5htR2_3raceF1 | GAAGGAAAGCCAATGAAGAAGATAGG | 3' RACE PCR (1211-1236) |
| 5htR2_3raceF2 | GTATTCGATTTTGTACATGGTTAGG | 3' RACE PCR (2134-2159) |
| 5htR2_3raceF3 | TAAAGTGTTCGACAAGCATTCAAG | 3' RACE PCR (2205-2229) |
| 5htR2_3raceR | TCTTGAATGCTTGTGCAAAACAC | 3' RACE PCR positive control (2230-2209) |
| 5htR2_5raceR1 | TGCACCAAACCTGTTGTAAGC | 5' RACE PCR (1047-1027) |
| 5htR2_5raceR2 | CGCTCGCCCAACC | 5' RACE PCR (835-823) |
| 5htR2_5raceR3 | GCATGACGAGTATGGCGAC | 5' RACE PCR (370-352) |
| 5htR2_5raceF | GCAGCTGGCAACATCC | 5' RACE PCR positive control (250-265) |
| 5htR2-ORF-F1 | GGCCAAGAAGAAGAGGATGTG | Amplification of complete open reading frame (153-173) |
| 5htR2-ORF-R1 | GTTATTGTTACATCTGCCTACGTTT | Amplification of complete open reading frame (2297-2273) |
| 5htR2-ORF-kozak | GCCACCATGTGCAAGTATACAAACAGG | Addition of Kozak translation initiation sequence into expression construct (169-188) |
| 5htR2-ORF-R2 | CTACGTTCTAGGTGTCCAGCG | Expression construct amplification (2280-2260) |
| 5htR2-qPCR-F | GAACAACGGCAGAAGCTTGG | Sense primer for qPCR located on exon 4 |
| 5htR2-qPCR-R | AATGCCCTCCTCTTTGTATGG | Anti-sense primer for qPCR located on exon 5 |

Initial oligonucleotide sequences designed based on high-scoring hits from tBLASTn search of preliminary *R. prolixus* genome database using *D. melanogaster* serotonin receptor type-2 sequences as query. Oligonucleotide sequences used in screening of cDNA plasmid library and RACE PCR based on partial initial sequence obtained. Final primer sets for verification of complete open-reading frame (ORF) and mammalian expression construct based on complete cDNA sequenced de novo and sequence comparisons with the preliminary *R. prolixus* genome assembly.

similarity to previously annotated serotonin receptors (Hauser et al., 2006) and recently classified as a serotonin receptor type-2B (Gasque et al., 2013). The *D. melanogaster* 5HTR-2B sequence was used for *in silico* screening of the *R. prolixus* genome (as described above) that yielded several high scoring matches. Similar to the *in silico* screen using the type 2A sequence as bait, many of the high-scoring candidate matches to the serotonin receptor type-2B localized to a single supercontig (GL563092) and so gene-specific primers (Sigma Aldrich, Oakville, ON, Canada) were designed to isolate a larger portion of this candidate *R. prolixus* 5HTR2 cDNA sequence (see **Table 1**) following PCR conditions similar to those described above using the ThermoPol Taq Polymerase (New England Biolabs, Whitby, ON, Canada). These efforts extended the partial cDNA sequence to a size of 2003 bp but which nonetheless remained incomplete on the 5' and 3' ends lacking start and stop codons, respectively.

In order to elucidate the entire open reading frame of the receptor, we screened a *R. prolixus* MT cDNA library implementing a PCR-based approach including *R. prolixus* 5HTR2b gene-specific primers (based on the incomplete 2003 bp partial cDNA sequence obtained above) and cDNA library plasmid-specific primers described previously (Paluzzi et al., 2010). In addition, to ensure we obtained the most complete cDNA encompassing both the coding sequence (i.e., open-reading frame) and the untranslated regions, we used a Rapid Amplification of cDNA Ends (RACE) approach following manufacturer suggestions (Roche Applied Science, Laval, QC, Canada) as described previously (Paluzzi et al., 2010). For 5' and 3' RACE, fresh cDNA was synthesized from 1 µg of total RNA isolated from a pool of fifth instar tissue samples including nervous, gastrointestinal tract and reproductive tissues. Finally, the complete open reading frame (ORF) sequence was amplified using Q5 high-fidelity DNA Polymerase (New England Biolabs, Whitby, ON) from freshly prepared fifth instar *R. prolixus* MT cDNA synthesized from 500 ng total RNA using iScript RT Supermix (Bio-Rad, Mississauga, ON) following manufacturer recommended conditions. Primers used to amplify the complete ORF are listed in **Table 1** and high-fidelity PCR cycling conditions were as follows: 98°C for 30 s for initial denaturation, 98°C for 8 s (denaturation), 65.5°C for 20 s (annealing) and 72°C for 60 s (extension) repeated for 35 cycles that was followed by a final extension at 72°C for 2 min. An aliquot of the PCR reaction was visualized using standard agarose-gel electrophoresis and the remaining PCR reactions were column-purified using the EZ-10 Spin Column PCR Products Purification Kit (Bio Basic, Markham, ON). Amplicons were A-tailed to facilitate T/A cloning into pGEM-T Easy (Promega, Madison, WI) sequencing vector as previously described (Paluzzi et al., 2015). Several independent clones were sequenced to ascertain base accuracy (Center for Applied Genomics, Hospital for Sick Children, Toronto, ON). Preparation of expression constructs in pcDNA3.1 mammalian expression vector was carried out similarly to that described previously (Paluzzi et al., 2010), using primers (see **Table 1**) incorporating the consensus Kozak translation initiation sequence (Kozak, 1986).

Receptor Functional Assay to Determine Ligand Specificity

The pcDNA3.1 Rhopr5HTR2b receptor construct was used for transient expression in a recombinant Chinese hamster ovary cell line (CHOK1-aeq) stably expressing the calcium-sensitive bioluminescent protein aequorin (Paluzzi et al., 2012). Controls using vector without the 5HTR2b insert were included to ensure the functional responses observed were specific to the transiently expressed *R. prolixus* 5HTR2b receptor. Approximately 24 h prior to transfection, CHOK1-aeq cells were seeded at high density into T-75 flasks and were subsequently transfected at either 90% confluence using XtremeGENE HP DNA transfection reagent (Roche Diagnostics, Laval, QC) or 70–80% confluence using Lipofectamine LTX (Life Technologies, Carlsbad, CA) following manufacturer recommendations. Approximately 36–48 h after transfection, cells were prepared for the luminescence assay as previously described (Paluzzi et al., 2010). Kinetic luminescent measurements were made using a VICTOR X Light luminescence plate reader (PerkinElmer, Woodbridge, ON) or a Synergy 2 multi-mode microplate reader (BioTek, Winooski, VT).

Putative ligands and other chemicals were purchased from local suppliers (Sigma-Aldrich, Oakville, ON) and included selected biogenic amines (serotonin, tyramine, octopamine, dopamine), agonists (α -methyl serotonin), and antagonists (ketanserin, mianserin, spiperone, cyproheptadine, gramine, propranolol), that were tested either in isolation or in combination as required for each particular experiment. Biogenic amines and agonists were made up in double distilled water at a stock concentration of 100 mM while antagonists were made up in ethanol at a stock concentration of 1 mM.

Sequence Analysis of the Rhopr5HTR-2b Receptor

The deduced protein sequence of the *R. prolixus* serotonin type-2b receptor was analyzed for predicted hydrophobic transmembrane domains using the HMMTOP transmembrane topology prediction server [http://www.enzim.hu/hmmtop/; (Tusnady and Simon, 2001)]. In addition, the deduced Rhopr5HTR-2b protein sequence was evaluated for potential phosphorylation sites using the kinase-specific phosphorylation prediction server, NetPhosK 1.0 (Blom et al., 2004).

The *R. prolixus* serotonin type-2b receptor identified here along with selected structurally related orthologous sequences previously identified in other organisms or predicted in genomic databases were used in a comparative investigation of their primary sequences using ClustalW in MEGA 6.0 (Tamura et al., 2013). To determine the relationship among the various serotonin receptors, including the sequence identified in the current study, phylogenetic analyses were performed using the neighbor-joining method (Saitou and Nei, 1987) and maximum-likelihood method based on the Jones-Thornton-Taylor matrix-based model (Jones et al., 1992), which produced trees that had nearly identical topologies. In order to verify the significance in the determined relationships between the serotonin receptor sequences examined in this analysis, we performed a bootstrap test with 1000 iterations (Felsenstein, 1985).

vessel activity during an initial incubation with saline compared to a second treatment with a given test compound.

Results

Cloning and Gene Structure of a *R. prolixus* 5HTR2b Receptor

Using a combined approach involving homology-based *in silico* screening of the preliminary *R. prolixus* genome data and PCR-based strategy to confirm and obtain further sequence information over the 5' and 3' regions using RACE PCR, we have identified a *R. prolixus* 5HTR2b (Rhopr5HTR2b) cDNA (Genbank accession KP325472). The total length of the Rhopr5HTR2b cDNA amplified is 2299 bp (Figure 1A), with no additional splice variants identified. Using Geneious 6.1.2, the cloned 2299 bp *R. prolixus* 5HTR2b cDNA was used for a local nucleotide BLAST search of the preliminary *R. prolixus* genome dataset to enable detection of exon-intron sites. The gene spans over 80.7 kb and includes six exons with lengths of ≥ 103 bp; 294, 233, 229, 340 bp and ≥ 1100 bp as well as five introns with lengths of 42,922, 22,799, 9137, 3152, and 401 bp (Figure 1B). The single open reading frame of 2109 bp produces a protein of 703 amino acid residues (Figure 1A) with an expected molecular mass of 78.7 kDa. In common with other members of the GPCR superfamily, the deduced protein sequence contains seven hydrophobic transmembrane domains. The amino-terminus (N-terminus) has a length of 16 amino acids while the carboxyl terminus (C-terminus) length is 25 amino acids. The open reading frame begins on the second exon, which also yields the first and second transmembrane domains. The third and fourth transmembrane domains are localized to the third exon while the fifth hydrophobic domain is found on the fourth exon. The fifth exon lacks any predicted transmembrane domains but yields the N-terminal region of the third intracellular loop while transmembrane domains six and seven are localized on the sixth and final exon.

Protein Sequence Analysis and Phylogenetics

Kinase specific phosphorylation sites were predicted using NetPhosK 1.0 (Blom et al., 2004) under a more stringent threshold cut-off ($=0.75$). A number of phosphorylation sites were predicted based on the deduced protein sequence (Figure 1A), although any functional significance awaits further study. Protein kinase A predicted phosphorylation sites include Thr₁₃₁, Thr₂₃₇, Ser₃₆₆, Ser₅₂₅, and Ser₅₅₄. Predicted protein kinase C phosphorylation sites in the Rhopr5HTR2b sequence include Thr₁₂₆, Thr₁₃₁, Thr₂₁₈, Thr₂₃₇, Ser₃₉₇, Ser₅₂₅, and Ser₅₅₉. Within the third hydrophobic transmembrane region, a highly conserved aspartate residue (Asp₉₃) is present and is commonly found in aminergic neurotransmitter GPCRs as it is necessary for interaction with the positively charged amine moiety of aminergic ligands permitting receptor-ligand binding (Kristiansen and Dahl, 1996; Kristiansen et al., 2000). Additionally, vertebrate 5HTR type-2 receptors contain a highly conserved serine residue that is localized in close proximity to Asp₉₃ (approximately one helical turn), which is a critical residue for interaction with the charged amine side chain of serotonin

(Almaula et al., 1996). Notably, this serine residue facilitating receptor-ligand interaction is absent in Rhopr5HTR2b as well as orthologs from other insects.

Utilizing the ClustalW plugin in Geneious software, the *R. prolixus* 5HTR2b deduced protein sequence was compared to highly similar type 2b receptors from other insects including *Tribolium castaneum* [Genbank accession DAA64510; (Hauser et al., 2008)], *A. mellifera* [Genbank accession CBX90121; (Thamm et al., 2013)] and *D. melanogaster* [Genbank accession AGP51353; (Gasque et al., 2013)]. High sequence similarity or identity was observed largely over the predicted transmembrane domains (Figure 2A). In addition, regions of high similarity and identity were also observed over intracellular and extracellular loops, which may indicate regions essential for intracellular signaling and ligand recognition, respectively. In addition to the potential phosphorylation sites discussed above, the C-terminus contains a conserved cysteine residue (Cys₆₉₂ in Rhopr5HTR2b) that may undergo post-translational palmitoylation (Thamm et al., 2013). The addition of palmitate groups could control multiple receptor functions (Gorinski and Ponimaskin, 2013) including receptor dimerization or G protein association (Zheng et al., 2012) since palmitoylation can create different intracellular loop arrangements with palmitate groups penetrating the lipid bilayer (Goddard and Watts, 2012). Phylogenetic analysis of the *R. prolixus* 5HTR2b receptor deduced protein sequence, which included a comparison to representative sequences from the three main families of insect serotonin receptors and structurally related vertebrate homologs, yielded a tree strongly supporting Rhopr5HTR2b as a member of the insect type-2b receptor subfamily (Figure 2B).

Transcript Expression Analyses of Rhopr5HTR2b

Quantitative PCR (qPCR) was used to examine the expression profile of the Rhopr5HTR2b receptor in fifth instar tissues. Highest transcript enrichment is found in the MTs and the salivary glands and lower levels, approximately three-fold less abundance, is found in the CNS, foregut, hindgut and dorsal vessel (Figure 3A). Expression was very low or absent in all other tissues examined which included midgut, reproductive tissues, prothoracic glands (with associated fat body) and a pool of tissue comprised of trachea, fat body, diaphragm, and abdominal nerves.

Considering the highest enrichment observed in MTs and the diuretic role of serotonin in the rapid post-feeding diuresis, we examined the expression profile between unfed and recently fed fifth-instar insects. We observed no changes in Rhopr5HTR2b transcript abundance between unfed and time points up to 6 h post-feeding (Figure 3B). Interestingly, expression is somewhat lower at 24 h post-feeding (by approximately 60%), but this was not significantly different from unfed insects (One-Way ANOVA, $p > 0.05$).

Cell Culture-based Receptor Functional Assay

In order to confirm this receptor as a *bona fide* target of serotonergic signaling, we utilized mammalian cell culture as a model to confirm the ligand and pharmacological sensitivity of this putative serotonin receptor. Recombinant CHOK1-aeq

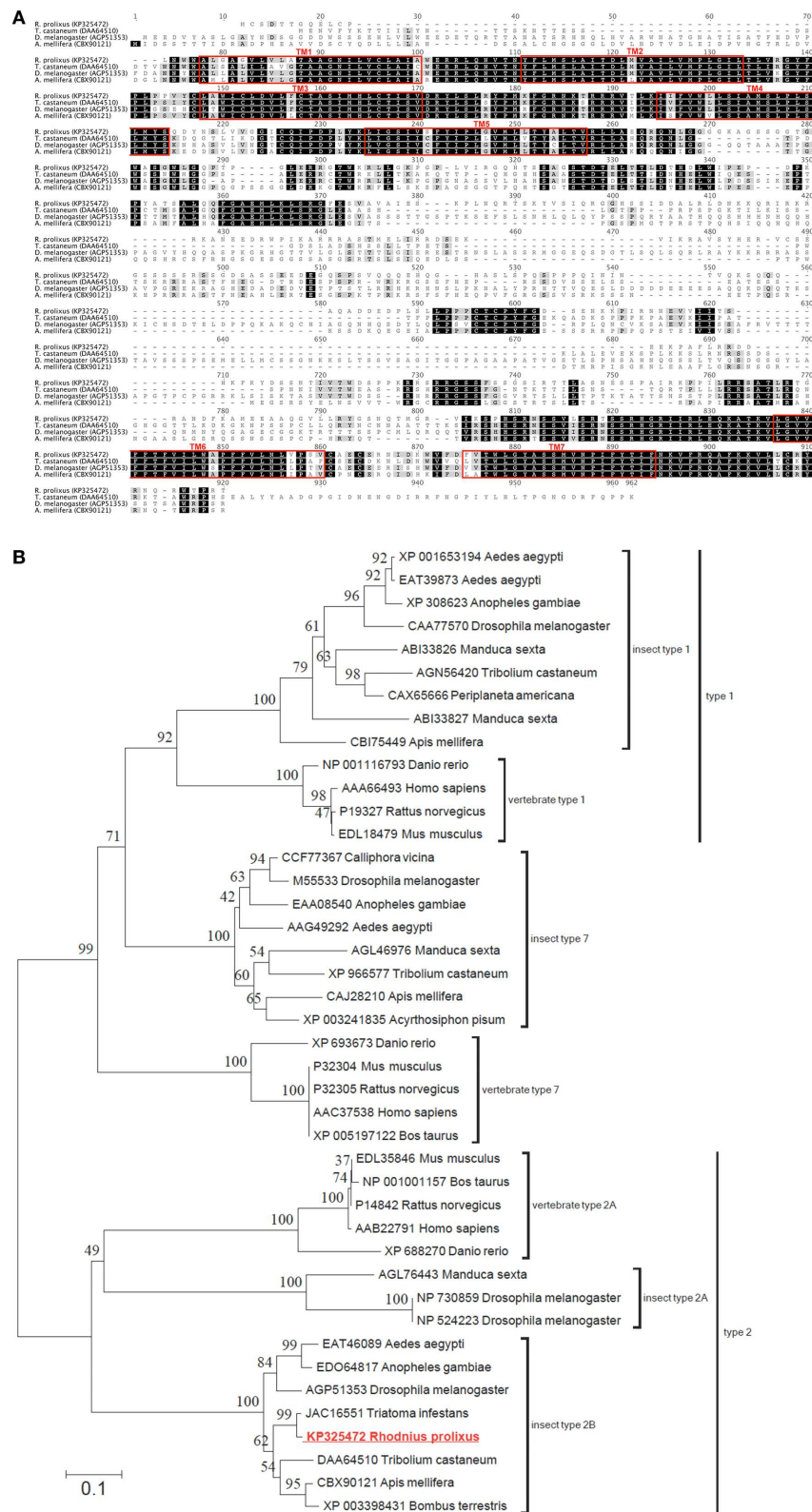


FIGURE 2 | Protein sequence alignment and phylogenetic analysis of selected serotonin receptors from insects and vertebrates. (A)

ClustalW alignment of selected serotonin type 2b receptors, with regions of

predicted transmembrane domains denoted by red outlined boxes. **(B)** Phylogenetic analysis using the neighbor-joining method of the deduced

(Continued)

FIGURE 2 | Continued

Rhopr5HTR2b receptor protein sequence based on the cloned cDNA. Branch lengths on the tree are representative of the number of average number of amino acid substitutions per site. Percent bootstrap support for the clustering of the related sequences is indicated by the numbers

adjacent to the nodes. Each protein sequence included in the analysis is identified by the GenBank accession number and the species name. The *R. prolixus* serotonin receptor identified in the current study (shown in red font) clusters within the clade including insect type 2b serotonin receptors.

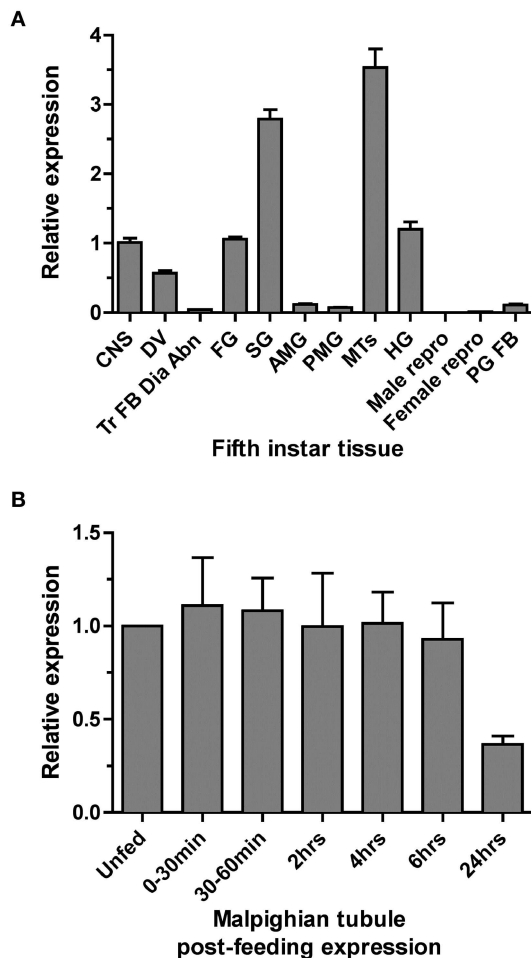


FIGURE 3 | *Rhodnius prolixus* serotonin receptor type 2b (*Rhopr5HTR2b*) transcript expression profile in fifth instar tissues determined by qPCR. (A) *Rhopr5HTR2b* transcript expression examined in fifth-instar tissues pooled from both male and female insects (except for sex-specific reproductive tissues). Relative expression is shown relative to levels in the CNS. (B) *Rhopr5HTR2b* transcript expression in MTs from unfed fifth instar and insects dissected at several time points up to 24 h post-blood meal engorgement. CNS, central nervous system; DV, dorsal vessel; Tr FB Dia Abn, trachea, fat body, dorsal/ventral diaphragm, and abdominal nerves; FG, foregut; SG, salivary gland; AMG, anterior midgut; PMG, posterior midgut; MTs, Malpighian tubule; HG, hindgut; PG FB, prothoracic gland and associated fat body; Male repro, male reproductive tissue; Female repro, female reproductive tissue.

cells stably expressing the jellyfish photoprotein apoaquorin (Paluzzi et al., 2012) were used for transient expression of the putative *R. prolixus* serotonin receptor (*Rhopr5HTR2b*) using the pcDNA3.1 mammalian expression vector. CHOK1-aq cells transiently expressing *Rhopr5HTR2b* yielded dose-dependent

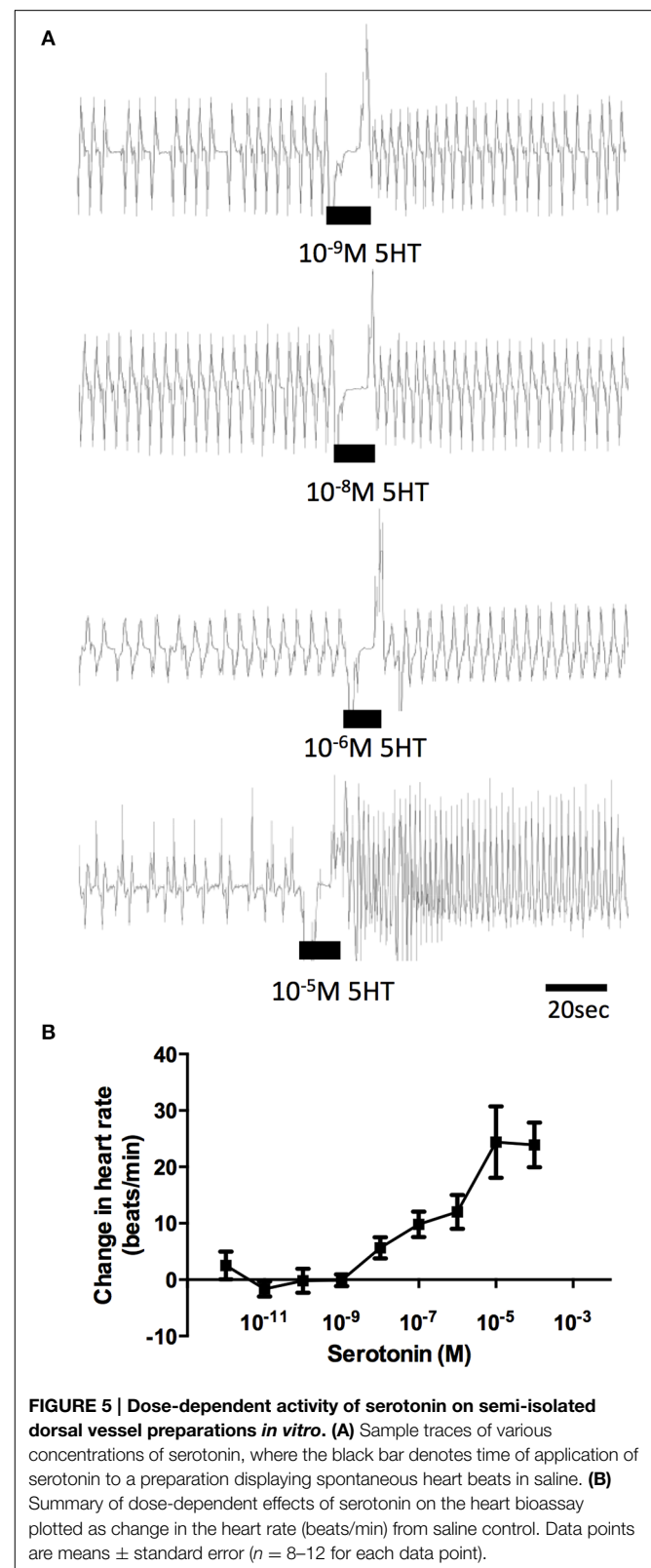
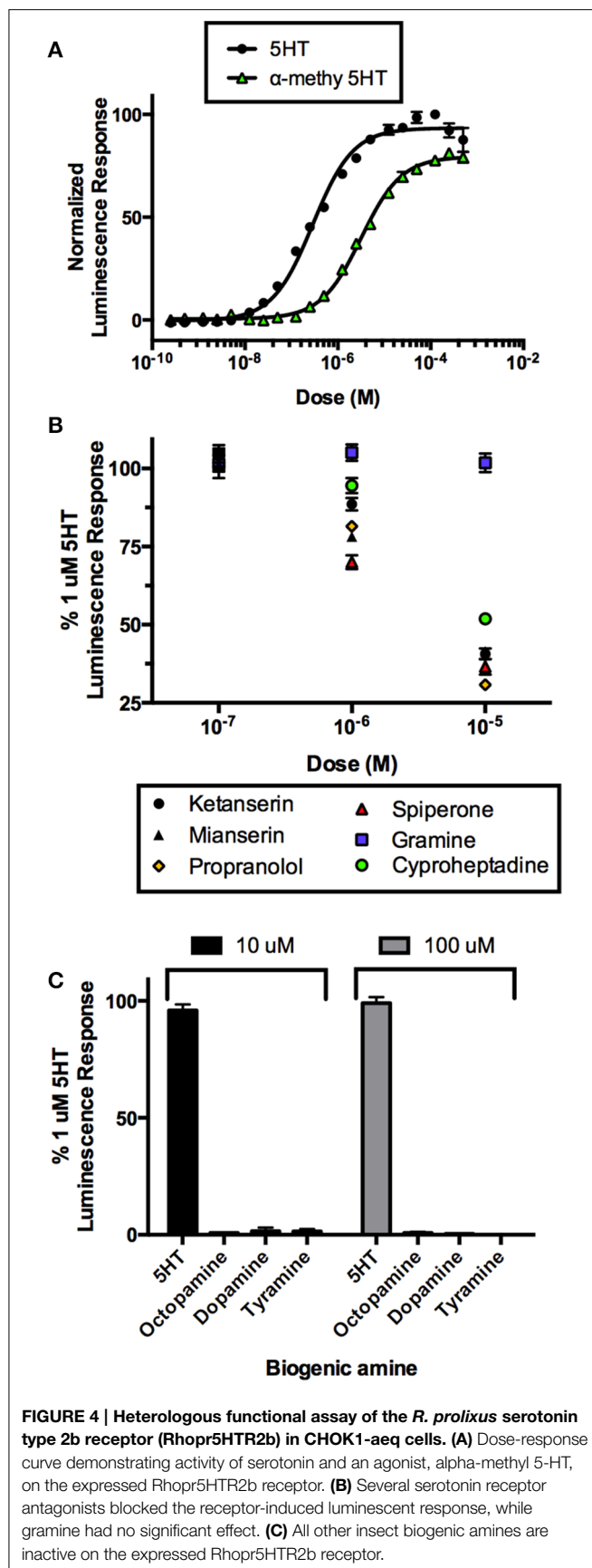
luminescence responses to serotonin (**Figure 4A**) with threshold activity in the low nanomolar range ($EC_{50} = 201 \text{ nM}$). We also evaluated the vertebrate serotonin receptor type-2 agonist, alpha-methyl serotonin, which similarly yielded a dose-dependent luminescence response, albeit with lower potency ($EC_{50} = 3.2 \mu\text{M}$). Application of serotonin or alpha-methyl serotonin to untransfected cells or cells transfected with empty vector (lacking the *R. prolixus* 5HTR2b cDNA) did not exhibit a luminescence response (data not shown).

In order to more fully characterize and better classify the receptor subtype, we tested a variety of known serotonin receptor antagonists in the presence of a sub-maximal dose of serotonin ($1 \mu\text{M}$). All drugs were ineffective at a dose 100 nM as luminescence output was not different from controls (**Figure 4B**). Lower doses were also tested but similarly showed no inhibitory activity (data not shown). Increasing the concentration of the putative antagonists to $1 \mu\text{M}$ showed differential drug sensitivity. Specifically, no change in luminescence response was seen in the presence of gramine but variable levels of inhibition (30–5% inhibition) were observed with spiperone, mianserin, propranolol, ketanserin, and cyproheptadine. At the highest tested concentration of antagonists ($10 \mu\text{M}$), gramine still had no effect on the luminescence response induced by serotonin whereas all remaining antagonists caused a greater reduction in the luminescence response (~ 70 – 50% inhibition) observed for propranolol, spiperone, ketanserin, mianserin, and cyproheptadine (**Figure 4B**).

We examined the specificity of the 5HTR2 receptor for its aminergic ligand, 5-HT. Comparing to the luminescence output of 5HTR2b-transfected CHOK1-aq cells treated with $1 \mu\text{M}$ 5HT, we tested other biogenic amines (**Figure 4C**) including dopamine, octopamine and tyramine at two doses (10 and $100 \mu\text{M}$) expected to be saturating on their respective natural receptor targets. Our receptor yielded no response to any of the other amines tested with the exception of its confirmed natural ligand, 5-HT.

Dorsal Vessel Contraction Assay

The effects of serotonin on semi-isolated dorsal vessel preparations confirmed a stimulatory effect on the frequency of heart contractions (**Figure 5A**). Serotonin dose-dependently increased the rate of dorsal vessel contractions (**Figure 5B**) with threshold in the low nanomolar range (1 – 10 nM) and maximal effect observed in the mid micromolar range ($10 \mu\text{M}$). The average frequency of dorsal vessel contractions under physiological saline alone was 14.96 ± 0.7 beats/min and the most potent dose of serotonin ($10 \mu\text{M}$) led to an increase in contraction frequency by 24.5 ± 4.4 beats/min, which represents an approximate 165% increase relative to control.



Given the sensitivity of the receptor isolated in this study to alpha-methyl serotonin (a serotonin type-2 receptor agonist) when examined in the cell culture-based receptor functional

assay, we also tested this compound on the dorsal vessel bioassay. We selected an intermediate dose of serotonin (100 nM), which revealed a doubling in the frequency of dorsal vessel contractions (Figures 5B, 6A). As can be seen, α -methyl serotonin was not an effective agonist on the serotonin receptor type facilitating an increased heart rate (Figure 6A). We did observe some minor increases in the frequency of dorsal vessel contractions (i.e., heart rate) at the highest doses tested (10 μ M); however, these were not significantly different from saline control treatments (Figure 6B).

In order to further elucidate the receptor type mediating the cardioacceleratory action in *R. prolixus*, we tested a subset of the receptor antagonists utilized in the cell culture-based receptor functional assay, namely mianserin, ketanserin and gramine. As shown in the sample traces and the summarizing bar graph, application of mianserin and ketanserin alone at a dose of 5 μ M did not significantly affect the heart rate (Figures 7A–C). As shown earlier, 100 nM serotonin significantly increased the heart rate (Figure 7C) and also significantly increased the heart rate in the presence of the candidate antagonists mianserin and ketanserin (Figures 7A–C). Thus, unlike the result observed in the receptor functional assay, these two candidate antagonists were not effective at blocking serotonin's cardioacceleratory activity. We also examined the effects of gramine, which was not an effective antagonist on the isolated receptor when assessed using the cell culture-based functional assay (Figure 4C). When tested on its own, 50 μ M gramine had no effect on heart rate. In the presence of 100 nM serotonin and 50 μ M gramine, only a small increase to heart rate was observed (Figure 7D), which was not significantly different from the saline control. Upon wash off with saline, heart rate decreased and subsequent application of 100 nM serotonin alone led to an increased frequency of dorsal vessel contractions (i.e., elevated heart rate). Unlike our observations of the isolated receptor in the receptor functional assay, gramine appears to be a weak antagonist of the receptor controlling serotonin's chronotropic activity on the dorsal vessel.

Discussion

Diuresis in *R. prolixus* is under the control of at least two diuretic hormones, which includes the biogenic amine serotonin and RhoprCRF/DH (Lange et al., 1989; Maddrell et al., 1991; Te Brugge et al., 2011). In addition to its established role in stimulating fluid secretion by upper MTs, serotonin has a number of additional effects on a variety of tissues in *R. prolixus* (Orchard, 2006, 2009) as well as in other insects (Molaei and Lange, 2003; Dacks et al., 2006a; Wang et al., 2013; French et al., 2014; Majeed et al., 2014) that are mediated through a neurotransmitter, neuromodulator or neurohormone mechanism. We have identified a serotonin receptor that shares sequence characteristics most consistent with the insect type 2b serotonin receptors described previously (Hauser et al., 2008; Gasque et al., 2013; Thamm et al., 2013). The genomic organization is similar to that described in other insects, having either six (Thamm et al., 2013) or seven exons (Hauser et al., 2008; Gasque et al., 2013); however, the transmembrane domains in the *R. prolixus* 5HTR2b gene have different exon localizations (for comparison see Hauser et al., 2006; Thamm et al., 2013).

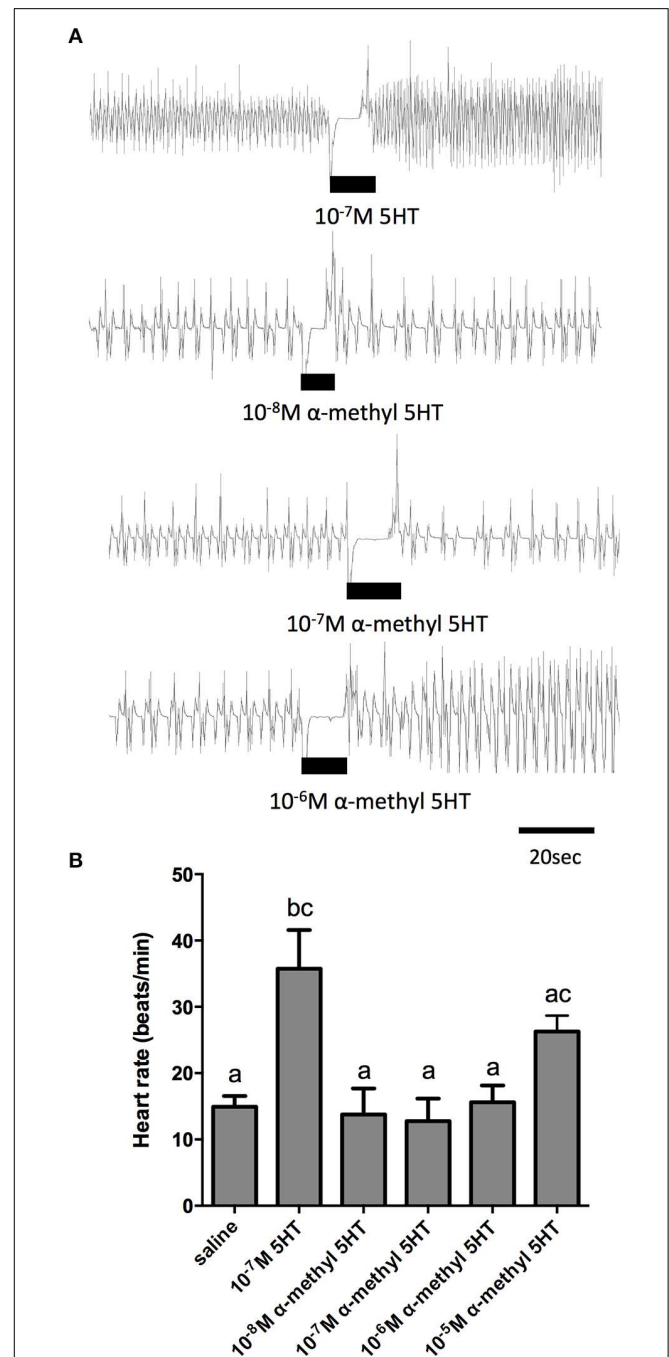


FIGURE 6 | The serotonin type 2 receptor agonist, α -methyl serotonin, is a weak cardioactive factor in *R. prolixus*. (A) Sample traces of 100 nM serotonin and selected concentrations of α -methyl serotonin. Black bars denotes application of serotonin or agonist at specified dose to preparations displaying spontaneous heart beats in saline. **(B)** Bar graph summary of the frequency of heart contractions (beats/min) under saline, serotonin or various concentrations of α -methyl serotonin. Columns marked with different letters are significantly different (ANOVA, Tukey-Kramer multiple comparison test, $P < 0.05$). Data points are means \pm standard error ($n = 8$ for each data point).

Phylogenetic analysis also supports the notion that the receptor isolated here belongs to the insect serotonin receptor type-2b subfamily—a nomenclature used to differentiate members of this

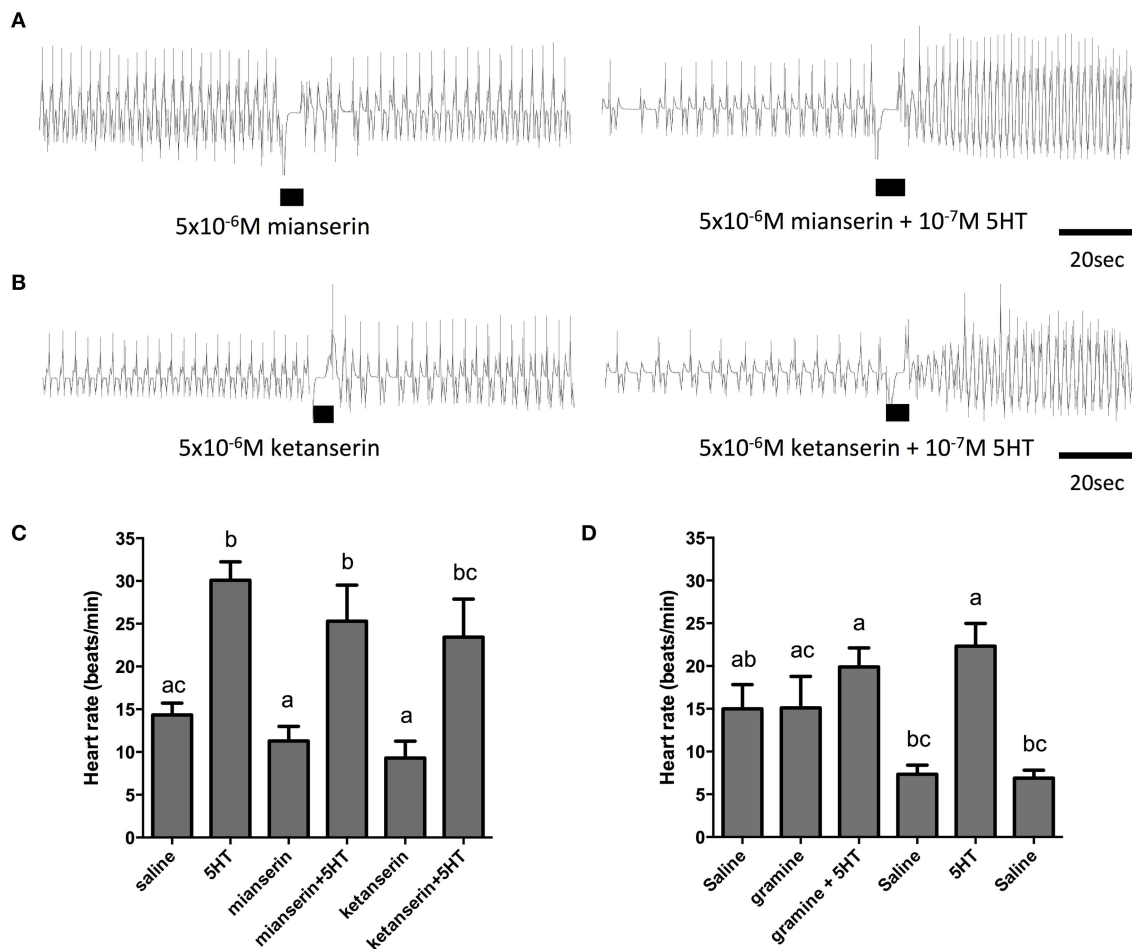


FIGURE 7 | The serotonin type 2 receptor antagonists, mianserin and ketanserin, are not effective inhibitors of serotonin-stimulated increases in heart rate. (A) Sample traces of 5 μ M mianserin application in isolation and in the presence of 100 nM serotonin. **(B)** Sample traces of 5 μ M ketanserin application in isolation and in the presence of 100 nM serotonin. Black bars denotes application of antagonist or antagonist with serotonin at specified concentrations, trace to the left of the black bar represents spontaneous myogenic contractions in saline and to the right of

the black bar is the response to serotonin or agonist application. **(C,D)** Bar graph summary of the frequency of heart contractions (beats/min) under saline, serotonin alone, single antagonists alone or in combination with serotonin. Columns marked with different letters are significantly different (ANOVA, Tukey-Kramer multiple comparison test, $P < 0.05$). Data points are means \pm standard error ($n = 6$ for each data point for assessments with mianserin and ketanserin; $n = 9$ for each data point for assessments with gamine).

subfamily from the insect serotonin receptor type-2a subfamily (Colas et al., 1995; Gasque et al., 2013).

Transcript expression profiling of *Rhopr5HTR2b* in various tissues of fifth instar stage *R. prolixus* revealed greatest enrichment in MTs and salivary glands. Pharmacological analyses in the heterologous system suggests this receptor could be the target of serotonin acting as a diuretic hormone enabling increased solute and water transport by the MTs following a blood meal. This notion is supported by previous studies that found ketanserin and spiperone are potent antagonists of serotonin-stimulated fluid secretion by MTs (Maddrell et al., 1991; Te Brugge et al., 2001), which were also the most active antagonists on the receptor we identified in this study. Interestingly, relative to the unfed animal, it appears there is no change in receptor transcript abundance over the first 24 h after blood meal engorgement, suggesting the Malpighian

tubule serotonin receptor that responds to haemolymph-borne serotonin is available in advance of feeding to ensure a prompt response to initiate the rapid post-feeding diuresis.

The pharmacological characteristics of the serotonin receptor type on *R. prolixus* salivary glands are not known. In *Calliphora vicina* salivary glands, different serotonin receptor types may regulate salivation since ketanserin is not effective at blocking serotonin-stimulated fluid secretion by isolated salivary glands (Maddrell et al., 1991), although serotonin-stimulated salivation in *C. vicina* is significantly inhibited by gamine (Berridge, 1972; Trimmer, 1985). Gamine was not effective at inhibiting the activation of the *Rhopr5HTR2b* receptor functionally analyzed here. Two receptors have been recently identified in blowfly salivary glands belonging to the serotonin receptor type-2a and type-7 classes (Roser et al., 2012), although both receptors displayed sensitivity to ketanserin

(Roser et al., 2012). A different receptor subtype could be expressed in the *R. prolixus* salivary glands where serotonin is delivered in the nerve supply and induces a dose-dependent increase in the frequency and amplitude of phasic muscle contractions (Orchard and Te Brugge, 2002). Serotonin has also been reported to dose-dependently stimulate secretion of saliva (Orchard, 2006).

Rhopr5HTR2b transcript expression was also detected in the CNS, dorsal vessel, foregut and hindgut of fifth instar *R. prolixus*. In the nervous system of insects, serotonin is known to play a variety of roles that includes regulation of feeding (Falibene et al., 2012; French et al., 2014), motor output and locomotion (Claassen and Kammer, 1986; Silva et al., 2014), control of clock neurons (Hamasa and Nassel, 2006; Kolodziejczyk and Nassel, 2011), and olfactory function (Mercer and Menzel, 1982; Kloppenburg et al., 1999; Dacks et al., 2006a; Tsuji et al., 2007; Kloppenburg and Mercer, 2008; Siju et al., 2008; Zhao and Berg, 2009; Watanabe et al., 2014). In *R. prolixus*, serotonin's distribution in the CNS is extensive, with approximately 150 neurons identified, whose projections and arborizations suggest that this neurochemical has a multitude of central as well as peripheral roles (Lange et al., 1988). A subset of the peripheral serotonin-like immunoreactive projections in *R. prolixus* are associated with regions of the gut including the foregut and hindgut (Orchard et al., 1988; Orchard, 2006). These regions of the gut also demonstrated *Rhopr5HTR2b* transcript enrichment, suggesting serotonin's roles could be mediated by this receptor in these tissues. In contrast, serotonin's action on the anterior midgut as a regulator of transepithelial transport (Farmer et al., 1981) and muscle contraction (Te Brugge et al., 2009) may not involve the identified receptor since no transcript was detected in this tissue. It is possible, however, that a closely related receptor (e.g., type 2a receptor class) may control these serotonergic actions in the anterior midgut since cAMP responsiveness to

serotonin showed a similar pharmacological profile, including sensitivity to the agonist α -methyl serotonin and inhibition by antagonists including ketanserin, mianserin and cyproheptadine, while the serotonin-induced increase of cAMP in the anterior midgut was not affected by spiperone (Barrett et al., 1993), one of the more active antagonists effective on *Rhopr5HTR2b*. Spiperone may be useful in discriminating between these closely related receptor subtypes.

The dorsal vessel is the primary circulatory organ in insects and is regulated by a variety of cardioactive factors, such as serotonin (Chiang et al., 1992; Zornik et al., 1999; Koladich et al., 2002; Dasari and Cooper, 2006; Feliciano et al., 2011). As previously reported (Chiang et al., 1992; Orchard, 2006), serotonin stimulates increases in heart rate in the low nanomolar range (threshold between 1 and 10 nM). In contrast to the pharmacological sensitivity of *Rhopr5HTR2b* as determined through the heterologous functional assay, the cardioacceleratory activity of serotonin had a unique pharmacological profile. High doses of the type 2 agonist, α -methyl serotonin, were not effective in significantly modifying the frequency of heart contractions. In addition, the dorsal vessel bioassay revealed insensitivity to ketanserin and mianserin. Taken together, these results suggest that at least two distinct serotonin receptors control critical physiological functions in this blood-feeding insect, one of which is *Rhopr5HTR2b*, involved in fluid secretion by the MTs. Other serotonin receptors appear to be involved in absorption by the anterior midgut and cardioacceleratory effects on the dorsal vessel.

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Tweaking the structure to radically change the function: the evolution of transthyretin from 5-hydroxyisourate hydrolase to triiodothyronine distributor to thyroxine distributor

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Often, we elucidate evolutionary processes backwards, starting with eutherian mammals and gradually climbing down the evolutionary tree to those species who have survived since long before mammals evolved. This is also true for elucidating the evolution of specific proteins, in this case, the protein currently known as “transthyretin” (TTR). TTR was first described in eutherian mammals and was known as a thyroxine (T4) binding protein. However, mammals are the exception among vertebrates in respect to the function of TTR, as in teleost fish, amphibians, reptiles and birds TTR preferentially binds triiodothyronine (T3), which is the active form of thyroid hormone (TH). The TTR gene possibly arose as a duplication of the transthyretin-like protein (TLP) gene, around the stage of the agnathans. Some vertebrate species have both the TTR and TLP genes, while others have “lost” the TLP gene. TLP genes have been found in all kingdoms. The TLPs analyzed to date do not bind THs or their analogs, but are enzymes involved in uric acid metabolism; specifically, they are 5-hydroxyisourate hydrolases. A *Salmonella* TLP knock-out strain demonstrated that TLP was essential for the bacteria's survival in the high uric acid environment of the chicken alimentary tract. Many other TLPs are yet to be characterized for their function although several have been confirmed as 5-hydroxyisourate hydrolases. This review describes the evolution of TLP/TTR and how subtle changes in gene structure or amino acid substitution can drastically change the function of this protein, without altering its overall 3D conformation.

Keywords: 5-hydroxyisourate hydrolase, mechanism, prealbumin, protein evolution, transthyretin, transthyretin-like protein, triiodothyronine, thyroxine

THE ROLE OF TRANSTHYRETIN IN THYROID HORMONE DISTRIBUTION

Thyroid hormones (THs) are involved in the regulation of growth, development and metabolism. There are two main forms of THs: 3',5',3,5-tetraiodo-L-thyronine (thyroxine, T4) and 5',3,5-triiodo-L-thyronine (T3) (**Figure 1**). The only site of TH synthesis is the thyroid gland, which secretes the THs (predominantly as T4) into the blood. THs are lipophilic and preferably partition into the lipid phase rather than the aqueous phase (1). To prevent the avid partitioning of THs into the membranes of the first cells they encounter, there are specific proteins in the blood that bind and distribute THs, thereby creating a circulating pool of sufficient size to distribute THs from their site of synthesis (the thyroid gland) via the aqueous environment of the blood stream to their sites of action, i.e., cells throughout the body (2, 3). In humans, there are three TH distributor proteins in the blood: albumin, transthyretin (TTR), and thyroxine-binding globulin. Of these three proteins, albumin is present in highest abundance but binds

THs with lowest affinity, thyroxine-binding globulin is present in lowest abundance but binds THs with highest affinity, and TTR is present in intermediate abundance and binds with intermediate affinity. Taking into consideration the affinities these proteins have for THs and the capillary transit times through tissues, albumin binds so weakly that the amount of TH that it delivers is extremely low; thyroxine-binding globulin binds THs so tightly, that the amount of TH it delivers is also low; whereas TTR binds THs with an intermediate affinity rendering it the most significant in terms of TH delivery to tissues [for a detailed quantitative analysis, see Richardson (4)]. This can be seen as analogous to the situation for Goldilocks and the Three Bears.

Once THs are bound to a TH distributor protein, with the affinity determined by the on and off rates, the TH can dissociate and enter a cell, either by diffusion (1) or via a membrane-bound TH transporter (5). Inside the cell, the TH can then be activated or inactivated by a family of enzymes called deiodinases (6). T4 is known as the “transport form” of TH, as it is the predominant form present in blood, whereas T3 is known as the “active form” of TH as it has higher affinity for the TH receptors (7), which are nuclear transcription factors. Thus, deiodinases can activate T4 to T3, or can inactivate T4 to reverse T3 (rT3) or

Abbreviations: TTR transthyretin, TLP transthyretin-like protein, 5-HIUase 5-hydroxyisourate hydrolase.

inactivate T3 to T2 (6). T3 can bind to cytosolic proteins and also to the thyroid hormone receptors (TRs), which can translocate into the nucleus, dimerize, recruit co-modulator proteins, and regulate transcription of specific genes. Many such genes are involved in growth, development, and metabolism, a spectacular example being metamorphosis of a tadpole (aquatic, herbivorous, gills, tail for locomotion) to a frog (terrestrial, carnivorous, lungs, four limbs

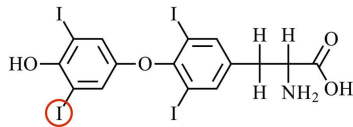


FIGURE 1 | The structure of the main thyroid hormone thyroxine.

Thyroxine (3',5',3,5-tetraiodo-L-thyronine; T4) is the predominant form of thyroid hormone secreted by the thyroid gland into the blood. T4 is converted to T3 (5',3,5-triiodo-L-thyronine) by an enzymatic removal of an iodine atom from the outer ring of T4. For example, the iodine atom circled in red. Two enzymes that perform this function are deiodinase 1 (e.g., in the liver) and deiodinase 2 (e.g., in the brain). T3 is the predominant form of thyroid hormone that binds to the nuclear thyroid hormone receptors.

for locomotion) [see Shi (8)]. Thus, TTR is a member of one of the five known classes of TH-binding proteins (TH distributor proteins; TH transporter proteins; deiodinases; cytosolic proteins; nuclear receptors) (**Figure 2**).

Transthyretin is a homo-tetramer, held together by non-covalent interactions, without post-translational modifications. Each subunit comprises eight β -strands that form two β -sheets and a short region of α -helix (**Figure 3**). The holo-protein has a central channel, which has two TH-binding sites (9); however, under physiological conditions only one site is occupied, due to negative co-operativity (10).

TTR NULL MICE HAVE A SUBTLY ALTERED PHENOTYPE, BUT HUMANS LACKING TTR HAVE NOT BEEN DESCRIBED

Mice in which the TTR gene has been inactivated [TTR null mice; (11)] have delayed development of several TH-regulated events including central nervous system development, growth of long bones, suckling-to-weaning transition (12), and adult TTR null mice have a hypothyroid phenotype in the central nervous system (13). Thus, the role of TTR in TH distribution in euthyrians has been demonstrated, despite having additional TH distributor proteins (albumin, TTR and thyroxine-binding globulin

Five classes of TH binding proteins:

1. THDPs (albumin, TTR and TBG)
2. TH transporters
3. Deiodinases
4. Cytosolic TH binding proteins
5. TH nuclear receptors

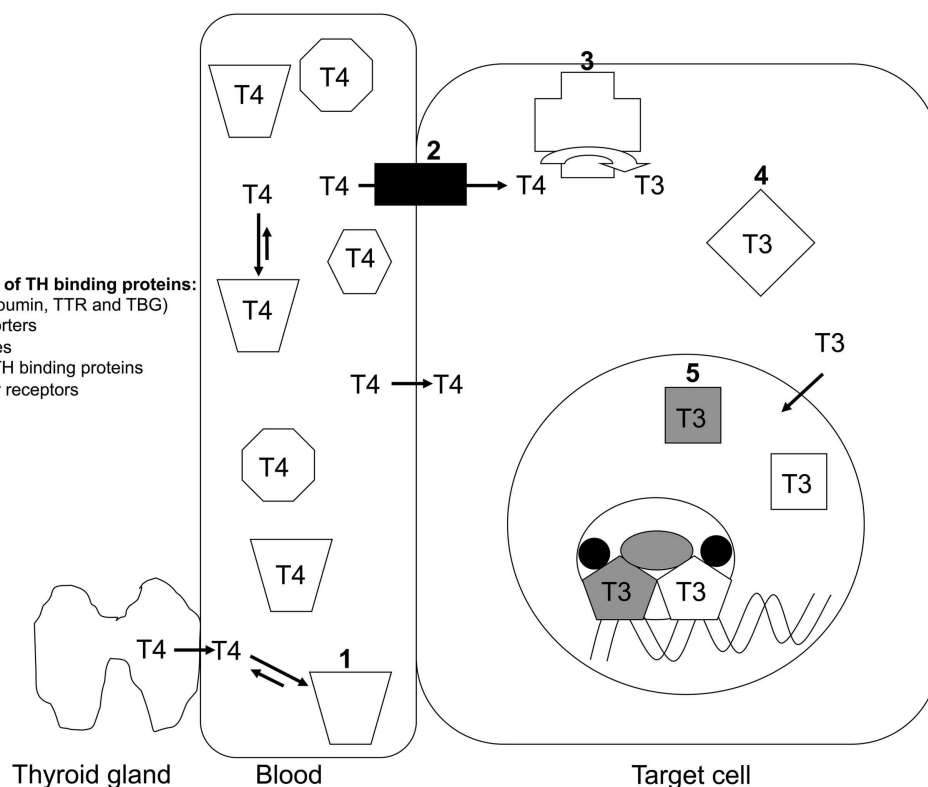


FIGURE 2 | The five classes of TH-binding proteins. T4 is the predominant form of TH secreted by the thyroid gland in mammals. T4 is the predominant form of TH in mammalian blood and T3 is also present in significant quantities, but for simplicity T4 is the only form of TH shown in blood in this schematic. More than 99% of TH (both T4 and T3) in blood is bound to a TH distributor protein (1) e.g. albumin, transthyretin (TTR) or thyroxine-binding globulin. TH can dissociate from its distributor protein

and enter cells via either TH transporters (2) or by diffusion. Inside the cell, THs can be deiodinated by a family of deiodinases (3), which can either activate (e.g. T4 to T3) or inactivate (e.g. T3 to T2) the TH. THs can bind to cytosolic proteins (4). Predominantly T3 binds to TH nuclear receptors (5), which bind to specific regulatory regions of TH-regulated genes and protein complexes to either promote or repress transcription of those genes (4).

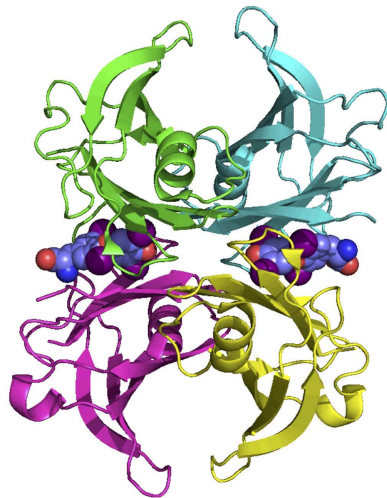


FIGURE 3 | The structure of human TTR. TTR is a homo-tetramer with a central channel that contains two potential thyroid hormone binding sites. Each subunit is rich in beta-sheet structure. Coordinates from Blake et al. (9).

in the blood are all synthesized by the liver and secreted into the blood). Humans lacking TTR have not been documented, although humans lacking albumin and thyroxine-binding globulin have been reported [see Harms et al. (14)]. Possibly, this is because TTR is the only TH distributor protein synthesized in the central nervous system: in the choroid plexus, which forms the blood–cerebrospinal fluid barrier. This TTR has been implicated in moving TH from the blood into the cerebrospinal fluid (1, 15, 16).

SHORTENING OF THE N-TERMINAL REGION OF TTR RESULTED IN CHANGING THE LIGAND FROM T3 TO T4

The amino acid sequence has been determined or derived from cDNA sequences for TTRs from more than 20 vertebrate species including teleost fish, amphibians, reptiles, birds, and mammals. The amino acid sequence has been highly conserved throughout vertebrate evolution, in particular, the amino acids which correspond to regions involved in monomer–monomer interactions, dimer–dimer interactions and those involved in TH binding [see Prapunpoj et al. (17)]. The region of TTR which has changed the most during vertebrate evolution is the N-terminal region, which has changed from longer (e.g. in amphibians) to shorter (e.g. in eutherian mammals). This has occurred in a step-wise manner, which implies a specific and persistent selection pressure acting on TTR during vertebrate evolution (Figure 4). Comparison of the cDNA with the genomic DNA in this region for each species revealed that the exon 1–exon 2 border was in the region of the gene corresponding to the N-terminal region of the protein subunit. Further analyses revealed that the position of the exon 1–intron 1 border did not change during vertebrate evolution. However, the position of the intron 1–exon 2 border appears to have shifted in the 3' direction, in a step-wise manner, due to a series of single base changes in the gene. Thus, the mechanism for the shortening of the N-terminal regions of TTRs can be explained by a series of

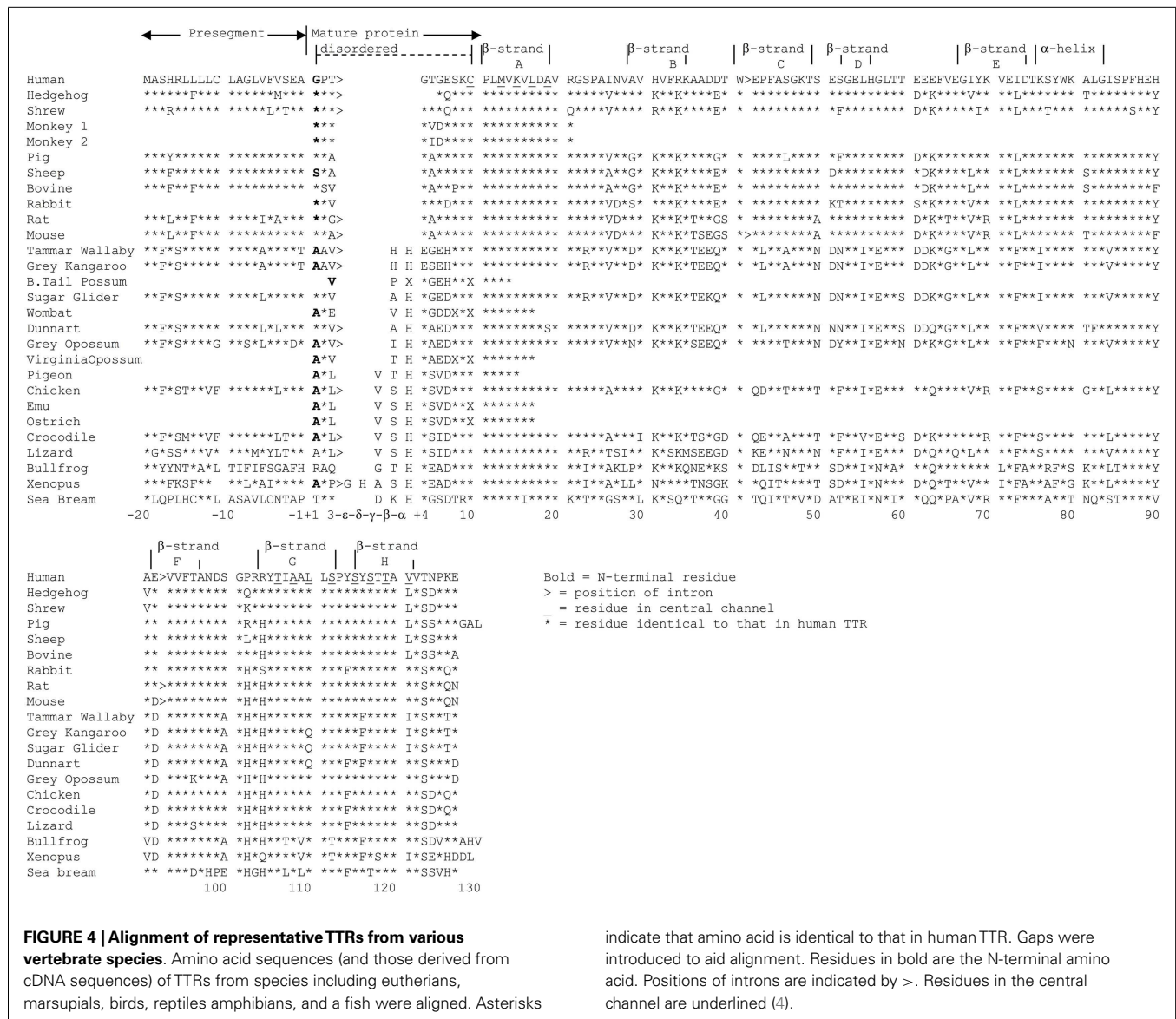
changes in the gene that “moved” an increasing number of bases from exon 2 into intron 1 (18) (Figure 5).

The effect of moving a series of bases of the TTR gene from exon 2 to intron 1 shortened the N-termini of the TTR tetramer but, most importantly, changed the function of TTR. Perhaps, this was the driving selection pressure for the movement of the intron 1–exon 2 splice site in the 3' direction.

Transthyretins with longer N-terminal regions (including those from teleost fish, amphibians, reptiles and birds) have higher affinity for T3 than for T4 (17, 19–22), whereas TTRs with shorter N-terminal regions (including those from marsupials and eutherians) have higher affinity for T4 (21) (Table 1). Thus, mammalian TTRs are the exception binding T4 > T3, as TTRs from all other classes of vertebrates bind T3 > T4. The hypothesis that binding of T3 or T4 was due to the N-terminal regions of TTRs was tested by two separate studies. The first study involved the purification of TTR from chicken blood followed by elucidation of the X-ray crystal structure of chicken TTR, to determine if there were structural changes in the TH-binding site between human TTR (which preferentially binds T4) and a TTR that preferentially bound T3. The structure of the TH-binding site in chicken TTR did not differ to that of human TTR (23). Thus, a different region of the molecule must be responsible and the best candidates were the N-terminal regions, which move freely in solution around the entrances to the channel containing the TH-binding sites. The second study involved a set of recombinant TTRs, including chimeric TTRs whose N-terminal regions had been swapped (e.g. N-terminal region of crocodile TTR attached to the “body” of human TTR and vice versa). These TTRs were analyzed for their affinities to T3 and T4. Indeed, the structure of the N-terminal regions did influence the affinity and preference of ligand binding (24).

Thus, by shifting the position of the intron 1–exon 2 border, TTR was able to change from being a T3 distributor to a T4 distributor. What could the selection pressure have been for changing the ligand of TTR from T3 to T4? T3 is the active form of the hormone whereas T4 is the pro-hormone. Perhaps, distributing the pro-hormone could be considered safer than distributing the active form of the hormone, requiring an additional level of activation of the pro-hormone by tissue-specific deiodinases, which are very tightly regulated in terms of developmental and tissue-specificity. In particular, this could be important in the central nervous system, as in (for example) the rat brain, the proportion of T3 generated by local deiodination of T4 is specific to the region e.g. 65% in the cortex, 51% in the cerebellum, 35% in the pons, 32% in the hypothalamus, 30% in the medulla oblongata and 22% in the spinal cord (25). Such tight regulation might not be possible if T3 were the predominant form of TH in the blood and cerebrospinal fluid.

To put this into context, we should consider the total T4 and total T3 levels in blood from various classes of vertebrates. A comprehensive review by Hulbert (26) has tabulated the concentrations of T3 and T4 in the blood of more than 80 vertebrate species (including several life stages for several species). In mammals, birds and reptiles, the circulating levels of T4 are higher than those of T3. However, in some amphibians and teleost fish, the levels for circulating T3 and T4 are similar, while in other species the levels of T4 are higher than those of T3. Of particular interest,

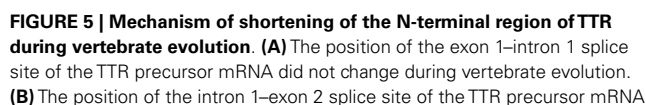


those animals undergoing metamorphosis or smolting have the characteristic peak in TH concentrations in their blood. In some species, this has been correlated with a transient expression of TTR (teleost fish; amphibian; reptile; polyprotodont marsupial) or TBG (diprotodont marsupial) (27). Speculation as to the driving force for greater control of T3 availability in specific brain areas in mammals, as opposed to other vertebrates is very tempting, but extremely speculative. Key TH-related features that distinguish mammalian brains from those of other vertebrates include (i) the corpus callosum and its extensive myelination and (ii) the highly developed cerebral cortex. Furthermore, mammalian fetuses have low levels of circulating T3 and maternal T4 (not T3) has a crucial role in brain development (28).

THE TTR GENE AROSE AS A DUPLICATION OF THE TLP GENE

As mentioned earlier, the amino acid sequence of TTR has been highly conserved during vertebrate evolution, to the extent that

the gene most likely arose prior to the divergence of vertebrates from non-vertebrates. Therefore, open reading frames which would code for transthyretin-like proteins (TLPs) were searched for and identified (17). Subsequently, as increasing numbers of genomes were sequenced, genes coding for more than 80 potential TLPs have been identified (and verified to be full-length and not to contain in-frame stop codons, etc.) in all kingdoms (29). Phylogenetic analysis revealed that TLP sequences generally clustered according to organism groups. Vertebrate TLPs clustered together, close to TTRs. TTRs were only found in vertebrate species, whereas TLP sequences were found in both vertebrate and non-vertebrate species (29). Analyses of the TLP gene sequences by signal-peptide prediction programs revealed that TLPs could be divided into three groups: (1) those without signal peptides were predicted to be cytoplasmic, which included most bacterial TLPs; (2) those with periplasmic localization signals, which were the enterobacteria; (3) those with peroxisomal



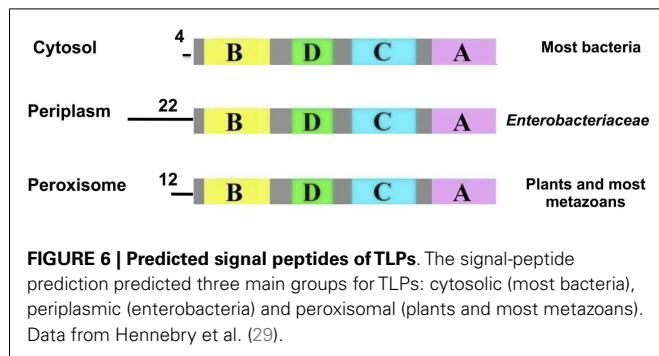
shifted in the 3' direction during vertebrate evolution. Double-headed arrows indicate the positions of splice sites. Consensus splicing site sequences are underlined. Asterisks indicate an identical base to that in human TTR precursor mRNA. N-terminal regions are boxed (19).

variety of sub-cellular localizations contrast with TTRs, which are secreted.

It was revealed by PCR that the TLP genes from a plant (*A. thaliana*), a worm (*C. elegans*) and bacteria (*E. coli* and *S. dublin*) were expressed in their respective species i.e. these open reading frames were genes that were expressed in nature. The respective cDNAs were cloned, sequenced and recombinant TLPs from these species were synthesized and found to be tetramers, similarly to TTR. However, these TLPs did not bind THs or TH analogs (29).

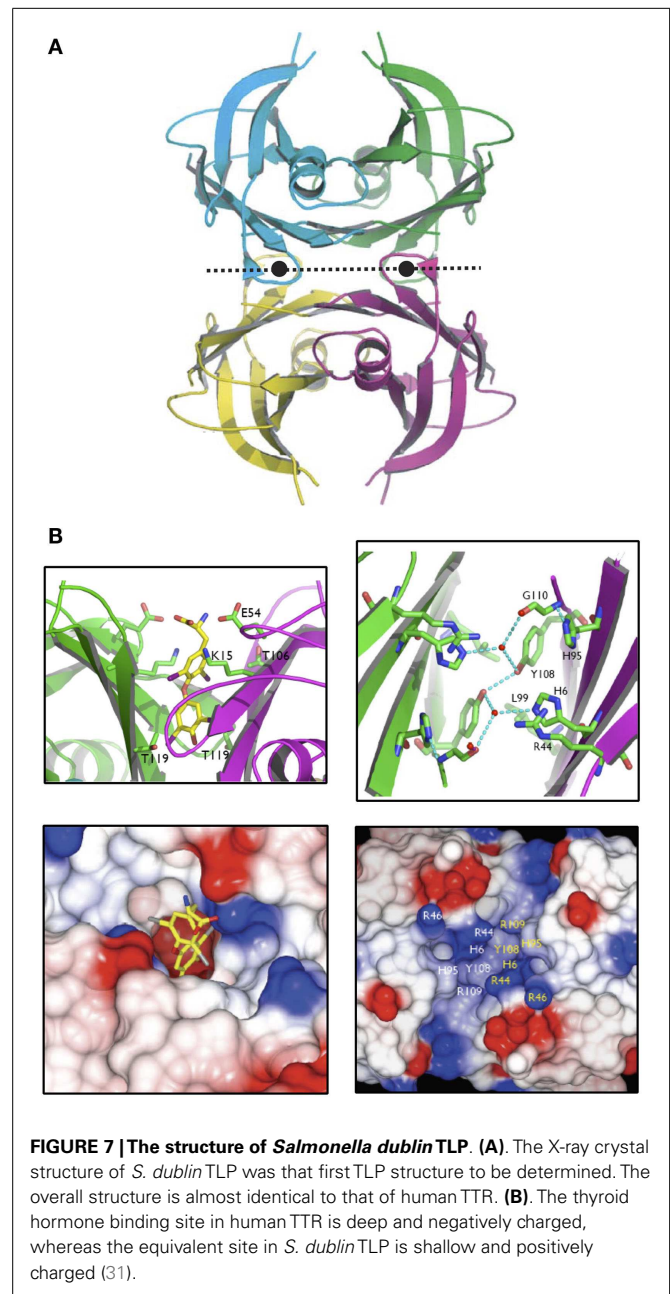
Table 1 | Comparison of affinities of TTRs for T4 and T3 (4).

| Source of TTR | Kd T4 (nM) | Kd T3 (nM) | Kd T3/Kd T4 |
|---------------|------------|------------|-------------|
| Eutherians | | | |
| Human | 13.6 | 56.6 | 4.2 |
| Sheep | 11.3 | 63.5 | 3.2 |
| Rat | 8.0 | 67.2 | 8.4 |
| Marsupials | | | |
| Wombat | 21.8 | 97.8 | 4.5 |
| Possum | 15.9 | 206.1 | 12.9 |
| Wallaby | 13.8 | 65.3 | 4.7 |
| Birds | | | |
| Emu | 37.4 | 18.9 | 0.51 |
| Chicken | 28.8 | 12.3 | 0.43 |
| Pigeon | 25.3 | 16.1 | 0.64 |
| Reptile | | | |
| Crocodile | 36.7 | 7.56 | 0.21 |
| Amphibian | | | |
| Toad | 508.0 | 248.0 | 0.49 |



The X-ray crystal structure of recombinant *Salmonella dublin* TLP was determined (**Figure 7A**) and was completely superimposable over those of vertebrate TTRs (31). The only differences were subtle changes in the region equivalent to the TH-binding site. Whereas in TTRs the binding site is deep and negatively charged, the equivalent position in TLP was shallow, elongated and positively charged, thereby preventing binding of THs (**Figure 7B**). Careful analysis of the operons within which several TLP genes were situated, allowed the identification of TLP in (at least several) bacteria as a 5-hydroxyisourate hydrolase (5-HIUase), involved in the oxidation of uric acid to allantoin (**Figure 8A**). This was confirmed experimentally for *S. dublin* TLP, including identification of some of the amino acids required for catalysis (**Figure 8B**) (31) and has also been confirmed for TLP in *Bacillus subtilis* (32), zebra fish (33), mouse (34), *E. coli* (35), *Arabidopsis thaliana* (36), *Klebsiella pneumonia* (37), Coelacanth (38), amphioxus (39) and rainbow trout (40).

An insightful paper by Cendron and colleagues (41) identified two amino acid substitutions that were most probably fundamentally critical for the modification of the TLP active site ablating enzymatic activity and allowing opening up of the central channel to allow binding of THs. These are Ile to Ala at position 16 of zebrafish TLP (corresponding to position 19 of human TTR)



and Tyr to Thr at position 116 of zebrafish TLP (corresponding to position 119 of human TTR). Li and colleagues demonstrated that in amphioxus (protochordate, close relative of vertebrates) TLP, the point mutation Tyr to Thr at position 156 (corresponding to position 116 in human TTR) was required for abolishing 5-HIUase activity and enabling T4 binding (39). While these mutations were probably instrumental in changing the landscape of the binding site from a shallow catalytic site to a deep channel, further minor mutations were probably required to optimize the channel for tighter binding of T3.

Interestingly, there are three splice variants of *Arabidopsis thaliana* TLP: two are cytoplasmic and one is located in the peroxisome (36). The peroxisomal isoform has a 5-HIUase domain

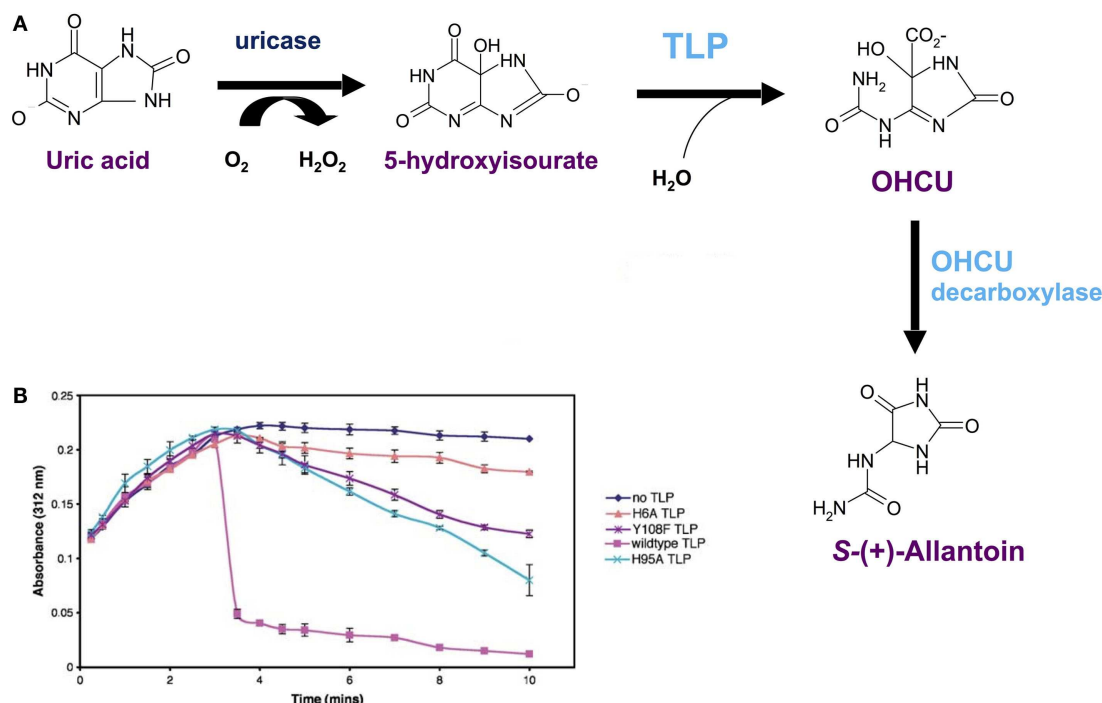


FIGURE 8 | *Salmonella dublin* TLP is a 5-hydroxyisourate hydrolase.

(A) The uricase pathway, responsible for the oxidation of uric acid to allantoin. **(B)** Hydrolysis of 5-hydroxyisourate (5-HIU) by *S. dublin* TLP and three mutated forms: H6A, H95A and Y108F. Uric acid was added to the solution containing

uricase and 5-HIU was monitored at 312 nm. Maximum 5-HIU was generated after 3 min and then spontaneously decomposed (black line). Addition of *S. dublin* TLP resulted in rapid hydrolysis of 5-HIU (magenta line). Rapid hydrolysis of 5-HIU did not occur for the mutants H6A, H95A and Y108F (31).

and an OHCU decarboxylase domain i.e. both enzymes occur in the one transcript resulting in a bi-functional enzyme. There is an internal peroxisomal signal peptide between the two domains (N-terminal to the OHCU decarboxylase domain), presumably targeting the bi-functional TLP to the peroxisome, where uric acid degradation occurs. The function(s) of the cytoplasmic TLPs are not yet known. Furthermore, teleost fish, whose genomes have undergone an additional whole genome duplication, have two forms of 5-HIUase. One form contains the peroxisomal signal peptide and the other does not (40). Thus, in organisms where there have been further TLP gene duplications, such as *Arabidopsis* and (at least some) teleost fish, there is further scope for neo-functionalization of the TLP gene products i.e. the protein resulting from the duplicated TLP gene could acquire a different function by acceptance of point mutations in the duplicated gene while the original TLP gene/protein remains unchanged.

IN *SALMONELLA*, TLP IS REQUIRED FOR SURVIVAL IN HIGH URIC ACID ENVIRONMENTS

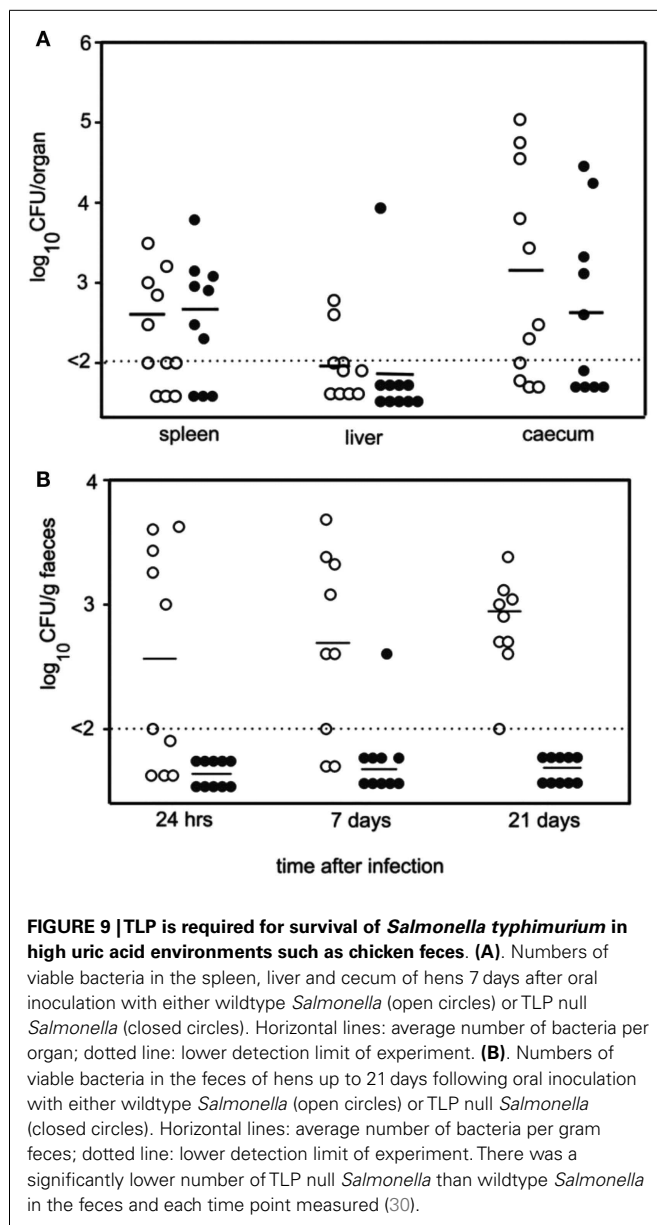
To demonstrate the function of *Salmonella* TLP in an animal model, a *Salmonella typhimurium* TLP knock-out strain was generated and its survival was compared with that of wildtype *Salmonella typhimurium*. The absence of TLP did not affect *Salmonella* survival in mice, whether the *Salmonella* were injected into the tail vein (and monitored for weight loss, development of enteric fever and bacterial load in liver or spleen) or infected orally (and bacterial load determined in Peyer's Patches, mesenteric lymph nodes,

liver and spleen) (30). Consequently, it was reasoned that if TLP was a 5-HIUase located in the periplasm of the *Salmonella*, then it would be important for the survival of the *Salmonella* in high uric acid environments such as the gastrointestinal tract of birds and reptiles (uric acid is present in high amounts in feces of reptiles and birds). Thus, mice might have been an inappropriate model animal for testing the effect of TLP on *Salmonella* survival.

Hens were inoculated with either the wildtype or the TLP knock-out strain of *Salmonella*. The feces of hens inoculated with the TLP knock-out strain of *Salmonella* contained significantly less live *Salmonella* than the feces of the hens inoculated with the wildtype *Salmonella* (Figure 9) (30). This demonstrated that TLP was important for the survival of *Salmonella* in high uric acid environments. Thus, not only is it important to choose the most appropriate animal model for such experiments, but this clearly showed that TLP is important in the survival of *Salmonella* in high uric acid environments.

MICE LACKING TLP SUFFER TOXICITY FROM URIC ACID OXIDATION INTERMEDIATES

Mice lacking TLP were generated and found to have increased thrombopoietin synthesis by the liver and enlarged livers (hepatomegaly) resulting in increased platelet counts in the blood (thrombocytosis). Most mice lacking TLP also developed hepatocellular carcinoma (42). It was concluded that this phenotype was due to the toxic uric acid oxidation intermediates resulting from the lack of 5-HIUase in the cytoplasm of the mouse hepatocytes.



CONCLUSION

TLP/TTR is an excellent model for the study of protein evolution. Notably because (i) it is found in all kingdoms, (ii) it has a stable structure, (iii) it lacks post-translational modification, (iv) it can be directed to many sites within a cell or secreted, (v) modification of just a few amino acids in the active site changed its function from a 5-HIUase to a T3 distributor, (vi) successive shifts in the position of the intron 1–exon 2 splice site changed TTR from a T3 distributor to a T4 distributor. Thus, apparently two different molecular mechanisms have resulted in changes in the functions of TLP/TTR. The functions of TLPs in plants and other organisms are likely to be different to those in *Salmonella* or mice. The differing signal peptides for the various groups of TLPs could be interrogated in conjunction with RNAseq analyses to gain insights into the functions of TLPs in various organisms. The suggestion

that cytoplasmic and periplasmic TLPs within a species evolved in separate pathways suggests divergence of functions between these groups of compartmentally distinct TLPs. Presumably, additional evolutionary mechanisms have also been used to modify the functions of TLPs in each compartment in these species. These mechanisms remain to be investigated.

ACKNOWLEDGMENTS

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Parasites and steroid hormones: corticosteroid and sex steroid synthesis, their role in the parasite physiology and development

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In many cases parasites display highly complex life cycles that include the penetration and permanence of the larva or adults within host organs, but even in those that only have one host, reciprocal, intricate interactions occur. Evidence indicates that steroid hormones have an influence on the development and course of parasitic infections. The host gender's susceptibility to infection, and the related differences in the immune response are good examples of the host-parasite interplay. However, the capacity of these organisms to synthesize their own steroidogenic hormones still has more questions than answers. It is now well-known that many parasites synthesize ecdysteroids, but limited information is available on sex steroid and corticosteroid synthesis. This review intends to summarize some of the existing information in the field. In most, but not all parasitosis the host's hormonal environment determines the susceptibility, the course, and severity of parasite infections. In most cases the infection disturbs the host environment, and activates immune responses that end up affecting the endocrine system. Furthermore, sex steroids and corticosteroids may also directly modify the parasite reproduction and molting. Available information indicates that parasites synthesize some steroid hormones, such as ecdysteroids and sex steroids, and the presence and activity of related enzymes have been demonstrated. More recently, the synthesis of corticosteroid-like compounds has been shown in *Taenia solium* cysticerci and tapeworms, and in *Taenia crassiceps* WFU cysticerci. In-depth knowledge of the parasite's endocrine properties will contribute to understand their reproduction and reciprocal interactions with the host, and may also help designing tools to combat the infection in some clinical situations.

Keywords: parasites, steroid synthesis, sex steroids, corticosteroids, steroidogenic enzymes, *Taenias*, cysticerci

Parasites and Host Interplay: Steroid Hormones Influence Parasite Development and Survival

Progesterone, androgens, and estrogens are present and have critical roles in the vertebrate reproduction and metabolism, but the influence and occurrence of steroid hormones in invertebrates had received less attention (Lafont, 2000) the interplay between the parasite and the host defines the intensity of parasite infections. In many cases, the presence of parasites

in the host changes its endocrine equilibrium due to the activation of the immune system response, which finally affects the endocrine system through the influence of cytokines and growth factors released by the immune cells. It is now widely accepted that corticosteroids and sex-related hormones influence the immune response (Roberts et al., 2001; Coutinho and Chapman, 2011; Reyes-Hernandez et al., 2013), thereafter any endocrine perturbation initiated by an infection will change the neuroendocrine equilibrium. These hormonal changes resulting from a spontaneous or experimental infection, affect the parasitic charge, the course of the infection and the parasite's survival (Barthelemy et al., 2004). On the other hand, some parasite infections disrupt the host endocrine system, in a noteworthy example, the dromedary bull, *Camelus dromedarius*, parasitized with *Tripanosoma evansi*, presents changes in the sexual steroid plasmatic concentrations, as well as in the semen characteristics (Al Qarawi, 2005). Furthermore, the male Fence Lizard (*Sceloporus occidentalis*) infected with the malarial parasite *Plasmodium mexicanum* shows several reproductive pathologies, such as fewer courtship and decreased testosterone levels (Dunlap and Schall, 1995) and the *Toxoplasma gondii* infection enhances testicular steroidogenesis in rats (Lim et al., 2013). Interestingly, it has been shown that the host hormonal environment determines the susceptibility, the course, and severity of many parasite infections, and therefore a clear dichotomy in infection susceptibility between males and females had been observed (Morales-Montor et al., 2004). A rich estrogen environment facilitates *Taenia crassiceps* cysticerci proliferation, blocking thus the P450-aromatase with fadrozole decreased parasite load (Morales-Montor et al., 2002). Parasites may also alter the host's reproductive behavior (Thompson and Kavaliers, 1994) as had been also shown in *Taenia crassiceps* ORF infected male mice (Morales et al., 1996).

Steroids and steroid synthesis inhibitors influence the fertility of *Schistosoma mansoni* *in vitro* (Morrison et al., 1986) while progesterone, androstenedione, and ecdysone increased the length of the larvae *Ascaris suum*, a roundworm swine parasite (Fleming, 1985), while progesterone, 17 β -estradiol, and testosterone added to the culture medium enhanced the number of *Plasmodium falciparum* gametocytes (Lingnau et al., 1993). Progesterone, but not testosterone decreased the *in vitro* molting process of *Trichinella spiralis* (Hernández-Bello et al., 2011) and *T. crassiceps* ORF cysticerci cell proliferation was increased by physiological concentrations of testosterone, and 17 β -estradiol added to the culture media (Romano et al., 2003), while high concentrations inhibited its reproduction (Escobedo et al., 2004).

The Role of Corticosteroids in the Host-parasite Interplay

It is well-known that non-physiological stress situations, such as social isolation, infections, persecution, etc., increase serum corticosteroids levels with the consequent impairment of the immune response. The interplay host-parasite is not the exception, for example social stress caused by female isolation increased blood *Typanosoma cruzi* infection in the

wild mouse *Calomys callosus*, which showed body weight loss and impaired immune response (Santos et al., 2008), whereas the hypothalamus-pituitary-interregal axis of rainbow trout was altered by the parasite haemoflagellate *Cryptobia* infection (Madison et al., 2013). In addition, the infection with *Anguillicola novaezealandiae* affects cortisol levels in the European Eel (Dangel et al., 2014). Frequently, the host and the parasites are affected in the course of infection, as in Bluegill Sunfish (*Lepomis macrochirus*) infected with *Utterbackia imbecillis*, which results in plasma cortisol increment in the host, favoring thus the parasite juvenile metamorphosis (Dubansky et al., 2011). On the other hand, *in vivo* treatment of rats with cortisol increased the growth rate of the protozoan parasite *Toxoplasma gondii* in isolated peritoneal macrophages (Wang et al., 2014).

Direct Effects of Glucocorticoids on Parasite Growth

Aside from the influence of corticosteroids in the course of parasitic infections, it had been shown that these hormones directly influence parasite's growth. For instance, cortisol and dexamethasone increase the *in vitro* multiplication of the haemoflagellate *Cryptobia salmonistica*, possible by their interaction with glucocorticoid receptor-like protein (Li et al., 2013, 2014). In this regard, dehydroepiandrosterone (DHEA) addition to the culture media decreased, while cortisol increased the *in vitro* growth and viability of *Entamoeba histolytica* (Carrero et al., 2006). We had shown that corticosterone and dexamethasone increase the capacity of *T. crassiceps* WFU cysticerci to synthesize androgens and estrogens, hormones that favor the parasite reproduction (Hinojosa et al., 2011).

Parasites Synthesize Steroid Hormones

Lipids and Steroid Hormones

Lipids, and particularly cholesterol and their metabolites, are required and synthesized by some parasites. Bansal et al. (2005) reviewed the requirement for lipids, particularly cholesterol, by pathogens like protozoa (Leishmaniosis, Malaria, and Toxoplasmosis). It has recently been stated that cholesterol exerts many of its functions by maintaining a specialized type of membrane domain called "lipid rafts" in a functional state. These domains are rich in cholesterol and sphingolipids and could be involved in signal transduction and in the entry of pathogens to the host cells (Simons and Toomre, 2000).

The incorporation and utilization of arachidonic acid, linoleic acid, 3-sn-phosphatidylcholine, tripalmitylglycerol, and cholesterol by adult *Schistosoma mansoni* was demonstrated by Rumjanek and Simpson (1980). These parasites exchange cholesterol and other metabolites during reproduction (Popiel and Basch, 1986; Silveria et al., 1986), while cholesterol is absorbed by the hydatid cysts of *Echinococcus granulosus* (Bahr et al., 1979). On the other hand, parasites like *Trypanosoma cruzi* and some species of *Leishmania* cannot use cholesterol but they synthesize ergosterol and related

24-alkylated sterols. Furthermore, *Leishmania* has a strict requirement for ergosterol for their survival and growth (Urbina et al., 2002; Magaraci et al., 2003; Bazin et al., 2006).

Ecdysteroids

Several parasites synthesize ecdysteroids, steroid hormones that are essential for arthropod molting; the capacity to synthesize these hormones has been used to classify cestodes (de Loof and Huybrechts, 1998). However, the role of these steroids in parasites remains obscure. Ecdysterone production has been reported in a variety of helminth species, among them, the nematodes *Dirofilaria immitis*, *Brugia pahangi*, *Ascaris suum*, and *Anisakis simplex*, the cestodes: *Moniezia expansa*, *Echinococcus granulosus*, and *Hymenolepis diminuta* and the trematodes: *Fasciola hepatica* and *Schistosoma mansoni* (Mendis et al., 1983, 1984; Fleming, 1985; Cleator et al., 1987; Evershed et al., 1987; Mercer et al., 1987a,b, 1990; Foster et al., 1992). In male and female, *Dirofilaria immitis*, and in female, *Ascaris suum* ecdysteroids were concentrated in the reproductive system, whereas the presence of ecdysteroids in the eggs of *Schistosoma mansoni* suggests a regulatory role in embryogenesis (Mercer, 1985). Ecdysteroids have also been found in cestodes like the sheep parasite *Moniezia expansa* and in the rat tapeworm *Hymenolepis diminuta* (Mercer et al., 1987b). Ecdysterone can be detected in the sera of infected hosts parasitized by *Schistosoma*, a trematode that synthesizes and releases the steroid to the host's circulation (Mercer, 1985). Although the role of ecdysteroids during insect metamorphosis had been widely demonstrated, the function of these steroids in parasites remains obscure.

Sex Steroids

Evidence for the steroidogenic capacity of *Schistosoma mansoni* was provided years ago by data showing the conversion of steroid precursors to its metabolites by the parasite homogenate (Briggs, 1972). We had shown that cultured *Trypanosoma cruzi* trypomastigotes synthesize androgens and estrogens from androstenedione and DHEA (Vacchina et al., 2008). We had also investigated the steroidogenic capacity of the cysticerci and tapeworm from *Taenia solium* and *Taenia crassiceps* WFU. The adult worm of *Taenia solium* and *crassiceps* WFU tapeworms are attached to the host gut with hooks that surrounds their head, and develop reproductive units called proglottids, where testis and ovaries gradually differentiate, and finally contain spermatocytes and infective eggs (Willms et al., 2003). *Taenia solium* cysticerci is the larval stage of the parasite and is found in the brain or muscle of humans and pigs, whereas *Taenia crassiceps* WFU cysticerci constitute a useful laboratory model due to their reproduction by budding in the peritoneal cavity of mice. We had been exploring the capacity of cysticerci to synthesize sex steroids *in vitro* and found that *T. solium* and *T. crassiceps* ORF cysticerci transform steroid precursors such as progesterone, dehydroepiandrosterone, and androstenedione to androgens and estrogens (Gómez et al., 2000; Jiménez et al., 2006; Valdez et al., 2006). Other experiments showed that drugs that block steroidogenic enzymes interfered the *in vitro* steroid

synthesis, suggesting the existence of this enzymes in the parasites (Aceves-Ramos et al., 2013).

Because of the presence of testis and ovaries in tapeworm proglottids, we had investigated the *in vitro* steroidogenic capacity of experimental *Taenia crassiceps* WFU and *solium* tapeworms obtained from the intestine of infected hamsters. *Taenia crassiceps* WFU tapeworms were incubated in the presence of ^3H -DHEA and tritiated androstenediol and 17β -estradiol where recovered from the culture media, which strongly suggest the presence and activity of enzymes from the $\Delta 5$ steroid pathway in these tapeworms (Fernández Presas et al., 2008). *Taenia solium* tapeworms kept in culture also synthesized sex steroids (Valdez et al., 2014).

Corticosteroids

Since the above mentioned literature indicated that *Taenia solium* and *Taenia crassiceps* cysticerci and tapeworms synthesized sex steroid hormones, we thought these organisms could also synthesize corticosteroids. Thereafter, we had incubated *Taenia crassiceps* cysticerci in the presence of ^3H -progesterone and found an important transformation into deoxycorticosterone (Valdez et al., 2012), a steroid that has mineralocorticoid functions in vertebrates and also display some glucocorticoid properties. In addition, the parasites synthesized corticosterone, which was measured by radioimmunoassay in the culture media. More recently, we found corticosteroid-like synthesis in *Taenia solium* tapeworms (Valdez et al., 2014). It had been shown that in the adrenal reticular zone of mammals an excess of progesterone can be inactivated by 20HSD activity (Pelletier et al., 2005) and that cortisol and corticosterone can be inactivated by 11β -hydroxylase in the adrenal glomerulosa zone (for a review see Odermatt and Atanasov, 2009) but to our knowledge these enzymes had not been investigated in *Taenia solium* or *crassiceps* WFU organisms. Besides their effects on the own parasite development and differentiation, the cysticerci, and tapeworm's steroidogenic capacity might play a role in the permanence of the parasites in muscle and brain tissues and in the host intestine.

Parasites Express Steroidogenic Enzymes

Some steroidogenic enzymes had been described in parasites. For instance, sterol-sterifying enzymes were found in *Toxoplasma gondii*, a protozoan incapable of cholesterol *de novo* synthesis (Lige et al., 2013). The presence of 3β -hydroxysteroid dehydrogenase in *Sarcocystis* spp had been shown by immunohistochemistry (Yarim et al., 2004); the enzyme is also present in *Taenia solium* cysticerci and tapeworms (Fernández Presas et al., 2008). Genes from the cholesterol synthesis pathway have been found and expressed in *Giardia intestinalis* (Hernandez and Wasserman, 2006). The parasite flatworm *Schistosoma japonicum* has a type 12 17-HSD that metabolizes estrone to estradiol (Zhou et al., 2009).

We have recently shown that *Taenia solium* cysticerci express the enzyme $17\beta\text{-HSD}$ that belongs to the short

chain dehydrogenases/reductase family (Aceves-Ramos et al., 2014). Transient transfection of HEK293T cells with Tso17 β -HSD-pcDNA3.1 (+) induced expression of Tso17 β -HSD that transformed ³H-androstenedione into testosterone. In contrast, ³H-estrone was not significantly transformed into estradiol. Therefore, *Taenia solium* cysticerci express a 17 β -HSD that catalyzes the androgen reduction and belongs to the short chain dehydrogenases/reductase (SDR) protein superfamily (Aceves-Ramos et al., 2014). Recently, a sequence (EmW000624600 which is available at: www.genedb.org/Homepage/Emultilocularis) with an identity of 84% with Tso1-17 β HSD and a total coverage has been described for *E. multilocularis*, suggesting the presence of 17 HSD enzymes in this *Taeniid* family (Tsai et al., 2012). However, the expression level and enzyme activity has not been yet investigated.

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Concluding Remarks

The knowledge of parasite endocrinology will contribute to our understanding of parasite biology and their interactions with the host. Sex steroids and corticosteroids are important hormones for the growth, differentiation, and performance in many species. Therefore, the synthesis of these hormones by parasites themselves may be critical for their own development and viability. In addition, it was shown that the production of steroids by parasites is regulated by corticosteroids and affected by steroidogenic inhibitors, which may be used as tools to combat the infection in some clinical situations. Furthermore, steroid synthesis by parasites may contribute to defend them from the attack of immune cells and therefore facilitate their survival in the host tissues.

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Bisphenol A and phthalate endocrine disruption of parental and social behaviors

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Perinatal exposure to endocrine disrupting chemicals (EDCs) can induce promiscuous neurobehavioral disturbances. Bisphenol A and phthalates are two widely prevalent and persistent EDCs reported to lead to such effects. Parental and social behaviors are especially vulnerable to endocrine disruption, as these traits are programmed by the organizational-activational effects of testosterone and estrogen. Exposure to BPA and other EDCs disrupts normal maternal care provided by rodents and non-human primates, such as nursing, time she spends hunched over and in the nest, and grooming her pups. Paternal care may also be affected by BPA. No long-term study has linked perinatal exposure to BPA or other EDC and later parental behavioral deficits in humans. The fact that the same brain regions and neural hormone substrates govern parental behaviors in animal models and humans suggests that this suite of behaviors may also be vulnerable in the latter. Social behaviors, such as communication, mate choice, pair bonding, social inquisitiveness and recognition, play behavior, social grooming, copulation, and aggression, are compromised in animal models exposed to BPA, phthalates, and other EDCs. Early contact to these chemicals is also correlated with maladaptive social behaviors in children. These behavioral disturbances may originate by altering the fetal or adult gonadal production of testosterone or estrogen, expression of ESR1, ESR2, and AR in the brain regions governing these behaviors, neuropeptide/protein hormone (oxytocin, vasopressin, and prolactin) and their cognate neural receptors, and/or through epimutations. Robust evidence exists for all of these EDC-induced changes. Concern also exists for transgenerational persistence of such neurobehavioral disruptions. In sum, evidence for social and parental deficits induced by BPA, phthalates, and related chemicals is strongly mounting, and such effects may ultimately compromise the overall social fitness of populations to come.

Keywords: EDC, bisphenol A, phthalate, xenoestrogen, rodent models, brain development, epigenetics, neuropeptides

INTRODUCTION

The perinatal environment can dramatically shape later adult behaviors, and these disruptions can be propagated through transgenerational transmission to future generations (Rosenfeld, 2014). The impact of varying parental investment on offspring brain development is also gaining interest (Dulac et al., 2014; Rilling and Young, 2014). Disturbances in parental behaviors, such as nursing/feeding, huddling over, and grooming the neonates can have dramatic epigenetic and phenotypic consequences that persist for generations to come (Weaver et al., 2006).

Due to underlying neural disruptions, parental and social behaviors can be impacted by developmental exposure to endocrine disrupting chemicals (EDCs). Strong conservation exists in brain development and function across species spanning from rodents to humans (Rice and Barone, 2000; Howdeshell, 2002). In animal models and humans, neurobehavioral development is vulnerable to EDCs, because of the organizational-activational of endogenous steroidogenic hormones.

One of the most preeminent discoveries in neuroendocrinology in the last century was that sex steroid hormones, testosterone and estrogen, guide perinatal brain development in a sex dependent manner (Phoenix et al., 1959; Arnold and Breedlove, 1985; Morris et al., 2004). This early brain programming is termed the “organizational effects” of these steroid hormones. However, full elaboration of many sex-dependent behaviors requires a later surge of testosterone in adult males (“activational effects”). The organizational-activational effects of testosterone in many brain regions are due to aromatization to estrogen (Watson and Adkins-Regan, 1989; Konkle and McCarthy, 2011). Exposure to EDCs that disrupt these normal steroidogenic effects may thus result in neurobehavioral deficits, including social and parental behavioral deficits.

The two most common EDCs associated with parental and social behavioral disturbances are bisphenol A (BPA) and phthalates. Therefore, these two will be the primary focus, although, where appropriate, effects of other EDCs on these traits will be discussed. BPA is one of the most widely prevalent EDCs

with production currently reported to be in excess of 15 billion pounds per year (Vandenberg et al., 2013; Grandviewresearch, 2014). BPA is present in a wide variety of commonly used products and applications, including polycarbonate plastics, the lining of metal food cans, certain dental sealants, thermal receipt paper, and many other items that are currently not required to be labeled that they contain BPA. The abundance of BPA has ensured extensive and longstanding exposure of animals and humans, including pregnant women (Environment Canada, 2008; Vandenberg et al., 2013). There is strong evidence that BPA is a neuroendocrine disruptor (Leon-Olea et al., 2014).

Di(2-ethylhexyl) phthalate (DEHP) is another ubiquitous EDC present in plastic products personal care products, paints, pharmaceuticals, food products, and textiles (Latini et al., 2003). Similar to BPA, this chemical can leach out of plastic products when heated. Its primary metabolite, mono-ethylhexyl-phthalate (MEHP) can also result in neuroendocrine disruption (Leon-Olea et al., 2014).

This review will first consider the evidence that BPA, phthalates, and other EDCs can alter parental care provided by the mother and the scant evidence to date by the father. Next, we will consider the impact of developmental exposure to these chemicals on social behaviors in animal models and linkages in humans. Finally, the potential mechanisms by which EDCs may affect these behavioral patterns will be explored.

EVIDENCE OF ENDOCRINE DISRUPTION OF PARENTAL BEHAVIORS

There have been no long-term studies examining for potential linkage of early exposure to these chemicals and later parental behavior deficits in humans. Therefore, this section will focus on the existing evidence from rodent and other animal models (Summarized in **Table 1**). A handful of rodent studies have reported that early exposure to EDCs can result in later effects on maternal care. General maternal behaviors assessed in these collective studies include the amount of time the female spends nursing, hunched over and in the nest, grooming, and latency to retrieve her pups.

CD-1 female mice exposed orally during the fetal and/or adult period to BPA (10 µg/kg body weight (bw)/day for gestational days 14–18) spend less time nursing their pups and huddling over them while in the nest (Palanza et al., 2002). Acute exposure of adult female Sprague-Dawley rats exposed to BPA (40 µg/kg bw, orally per day) from mating to weaning of their pups disrupts both active and passive maternal care, manifested by decreased time licking and grooming the pups, a trend to less ano-genital licking, and less time assuming an arched-back posture to allow the pups to suckle (Della Seta et al., 2005). Similarly, Wistar female rats treated with 5 µg BPA/kg bw/day from the first day of gestation through lactation (postnatal day, PND 21) decreased the amount of time they nursed and attended to their pups (Boudalia et al., 2014). Another study tested the effects of gestational exposure (0–19 days) to three oral doses of BPA (2, 20, and 200 µg/kg bw/day) on maternal care provided by BALB/c mice. In contrast to the prior studies, the highest dose of BPA tested increased the amount of time females spent licking and grooming and

nursing their pups in an arched back position (Kundakovic et al., 2013).

These conflicting rodent model findings may be attributed to various factors. First, all four of these above studies used rats or mice of different strains, and there is evidence of strain-specificity and phenotypic differences in response to BPA and other estrogenic EDC (Spearow et al., 1999, 2001; Kendzioriski et al., 2012). The administered dose and timing, duration of exposure (perinatal vs. adult), and corresponding generational differences (F_0 vs. F_1) may also be potential factors. Finally, differences in animal husbandry (composition of the cages and water bottles, diet-phytoestrogen or non-phytoestrogen free, and shavings-corn cob or aspen) might account for the disparate rodent results.

Infants may influence, through vocalizations, direct contact, and other forms of communication, the amount of parental care provided to them with males tending to initiate and receive more parental investment (Della Seta et al., 2005; Hao et al., 2011). Early EDC exposure though may alter an infant's ability to stimulate maternal care provided to them. For instance, the behavioral patterns of *Cynomolgus* monkeys (*Macaca fascicularis*) male infants prenatally (gestational day 20 through term) exposed to BPA (10 µg/kg bw/day via osmotic pump) were more reminiscent of females, and these mothers nursed their “feminized” sons less than those rearing control males (Nakagami et al., 2009).

While there are no studies to date on whether acute and developmental exposure to phthalates affects maternal care, other EDCs have been reported to disrupt these behavioral patterns. Long-Evans (LE) rat pups prenatally exposed through their biological dam to the environmental chemical 3, 4, 3', 4'-tetrachlorobiphenyl (PCB 77, 2000 µg/kg bw/day on gestational days 6 through 18 via daily subcutaneous injections) resulted in their foster dams spending an increased frequency nursing them and allogrooming (Cummings et al., 2005). This cross-foster approach further revealed the complex interactions exist between maternal and fetal exposure to PCB 77 and amount of time foster dams spent in the nest and grooming the pups, along with decreased duration engaged in high-crouch nursing. Perinatal exposure (either 3 days after the dam was paired with a male or 3 days after parturition of a previous litter through weaning of pups) via daily maternal oral dosing to diethylstilbestrol (DES, 0.2 µg/kg bw/day) or methoxychlor (MXC, 2000 mg/kg bw/day) of monogamous female pine voles did not however affect their later maternal care behaviors (Engell et al., 2006).

The impact of EDCs on later paternal care provided is largely unknown, probably because few mammalian species exhibit biparental care (Clutton-Brock, 1989). Yet, disturbances in paternal care may have dramatic epigenetic and phenotypic consequences that persist for several generations (Bredy et al., 2007; Braun and Champagne, 2014; McGhee and Bell, 2014). Even so, there have been no published rodent studies to date on how early exposure to EDCs affects paternal care. However, in polygynous sand gobies (*Pomatoschistus minutus*), where the male is responsible for tending the eggs, endocrine disruption of paternal care behaviors has been reported (Saaristo et al., 2010). Polygynous sand goby males compete for females and then assume responsibility for nest building and attendance. However, adult males

Table 1 | Animal model and human epidemiological studies linking bisphenol A (BPA) and phthalates exposure to parental and social behavioral changes.

| Publication(s) | Animal model/human cohort population | EDC(s) tested or correlation analysis performed | Dosing regimen/method to measure BPA concentrations | Major findings |
|---------------------------|--------------------------------------|---|---|--|
| PARENTAL BEHAVIORS | | | | |
| Palanza et al., 2002 | CD1 female mice | BPA | Prenatal exposure of F ₁ offspring to 10 µg BPA/kg body weight (bw)/day or oil by oral administration through the F ₀ dam from days 14 to 18 of gestation. Some F ₁ adults (2–2.5 months of age) were also treated with 10 µg BPA/kg bw/day or oil and treatments spanned from days 14 to 18 of gestation. There were thus those F ₁ offspring who only received prenatal or adult exposure to BPA and those that were exposed to this chemical during the prenatal and adult periods resulting in four different treatment groups. | <ul style="list-style-type: none"> • F₁ females exposed to BPA during the prenatal or adult period spent less time nursing and huddling over the pups but greater time engaged in nest building than controls and those that received BPA during both time periods. |
| Della Seta et al., 2005 | Sprague-Dawley female rats | BPA | Adult F ₀ exposure to 40 µg BPA/kg bw/day by oral administration from the day after mating (gestation) through lactation (42 day duration). | <ul style="list-style-type: none"> • Adult BPA exposure of F₀ dams disrupts passive and active maternal care. • These F₀ females spend decreased time licking and grooming pups and a trend to less ano-genital licking. • These F₀ dams had reduced duration of time assuming an arched-back posture, which allows pups to suckle. |
| Boudalia et al., 2014 | Wistar female rats | BPA | F ₀ adult exposure to 5 µg BPA/kg bw/day orally from the first day of gestation (GD1) through lactation (Post-natal day, PND 21). F ₁ offspring then treated every two days with same dosing solution as received by the F ₀ mother from weaning (PND 21) until mating at adulthood (PND 100). | <ul style="list-style-type: none"> • F₀ adult BPA exposed dams did not demonstrate any differences in incidence of resting inside or outside of the nest. • Lifelong BPA exposure of F₁ dams though resulted in them spending considerably less time outside of the nest. • F₀ dams did not demonstrate any differences in nursing position • F₁ dams spend less time assuming “arched” and “blanket” nursing positions. |
| Kundakovic et al., 2013 | BALB/c mice | BPA | Adult exposure of F ₀ dams to one of the three oral doses of BPA (2, 20, 200 µg/kg bw/day) from gestational days 0 to 19. | <ul style="list-style-type: none"> • Adult F₀ females exposed to the highest dose of BPA spent more time licking, grooming, and nursing their pups in an arched back position. • No effects were reported with the lower two doses. |

(Continued)

Table 1 | Continued

| Publication(s) | Animal model/human cohort population | EDC(s) tested or correlation analysis performed | Dosing regimen/method to measure BPA concentrations | Major findings |
|--|---|---|--|--|
| Nakagami et al., 2009 | Cynomolgus monkeys (<i>Macaca fascicularis</i>) | BPA | Male F ₁ infants exposed from gestational day 20 through term to 10 µg BPA/kg bw/day via osmotic pump. | <ul style="list-style-type: none"> • BPA-exposed F₁ male infant behavioral patterns in initiating maternal care became more reminiscent of female infants and subsequently these males were nursed less than control males. • F₁ females were not examined in this study. |
| SOCIAL BEHAVIORS: ANIMAL MODELS | | | | |
| Williams et al., 2013 | California mice (<i>Peromyscus californicus</i>) | BPA | Two weeks prior to breeding of the F ₀ dam through weaning at PND 30, they were exposed to 50 mg BPA/kg feed weight in the diet. F ₁ males and females were placed on a control diet at weaning through adulthood. | <ul style="list-style-type: none"> • Perinatally BPA exposed F₁ males show reduce territorial marking behavior when a control male was present in the testing arena. • Perinatally BPA exposed F₁ females showed decrease exploratory and increased anxiety-like behaviors. |
| Ward and Blum, 2012 | Native blacktail shiner fish (<i>Cyprinella venusta</i>) and introduced red shiner fish (<i>C. venusta</i>) | BPA | Adult F ₀ exposure for 14 days to BPA 1280 µg/L water (5.6 µM). | <ul style="list-style-type: none"> • Adult BPA exposure of F₀ fish disrupted normal visual communication signals and abolished species-dependent sexually selected behavioral traits. • Hybridization occurred between these two otherwise behaviorally isolated species. |
| Jasarevic et al., 2011 | Deer mice (<i>P. maniculatus bairdii</i>) | BPA | Two weeks prior to breeding of the F ₀ dam through weaning at PND 21, they were exposed to 50 mg BPA/kg feed weight in the diet. F ₁ males and females were placed on a control diet at weaning through adulthood. | <ul style="list-style-type: none"> • F₁ control and perinatally BPA-exposed female selectively rejected F₁ males prenatally exposed to BPA. |
| Razzoli et al., 2005 | Pair-bonded monogamous Mongolian gerbils (<i>Meriones unguiculatus</i>) | BPA and EE2 | Adult F ₀ females treated daily with oral administration of BPA (2 or 20 µg/kg bw/day) or EE2 (0.04 µg/kg bw/day) from day of pairing to day 21 of cohabitation. | <ul style="list-style-type: none"> • BPA and EE2 adult exposed F₀ females showed increase investigation of their F₀ control male partner and reduced exploration. |
| Wolstenholme et al., 2013 | C57Bl/6 mice | BPA | Prenatal exposure (7–10 days prior to F ₀ female being paired with a breeder male and for a 2 week duration) and ancestral exposure to 5000 µg/kg fw through the F ₀ maternal diet. | <ul style="list-style-type: none"> • Prenatal and transgenerational exposure to BPA increased the amount of time juvenile F₁ and F₃ male and female mice spent investigating a novel animal. • F₃ females ancestrally exposed to BPA persisted in investigating novel females, suggestive of impaired dishabituation. |

(Continued)

Table 1 | Continued

| Publication(s) | Animal model/human cohort population | EDC(s) tested or correlation analysis performed | Dosing regimen/method to measure BPA concentrations | Major findings |
|-----------------------------|--|--|--|--|
| Porrini et al., 2005 | Sprague-Dawley rats | BPA | Daily oral administration of 4000 µg BPA/kg bw/day to adult F ₀ dams from gestation through lactation (Mating through weaning of pups at PND 21). F ₁ females were not exposed to BPA after weaning. | <ul style="list-style-type: none"> • Perinatal exposure of F₁ females exhibited reduced amount of time playing with males and engaging in social grooming. • Findings are suggestive that developmental exposure to BPA may defeminize select social behaviors. • F₁ BPA exposed males were not assessed in this study. |
| Dessi-Fulgheri et al., 2002 | Sprague-Dawley rats | BPA | Perinatal exposure of F ₁ females to BPA either at 40 mg/kg bw/day via daily maternal (F ₀) oral dosing from conception to weaning or 400 mg/kg bw/day from gestational day 14 to postnatal day 6. | <ul style="list-style-type: none"> • Perinatally BPA-exposed F₁ females, especially those exposed to the low dose, demonstrated a masculinized response in play behavior, as evident by their preference in playing with and engaging in socio-sexual exploration of other females. • Perinatally BPA-exposed F₁ males showed increase play behavior with females. |
| Wolstenholme et al., 2011b | C57Bl6J mice | BPA | Gestational exposure of F ₁ offspring to 1250 µg BPA/kg fw in the maternal diet. | <ul style="list-style-type: none"> • Gestational exposure of both F₁ sexes resulted in increased play solicitations and approaches. |
| Jones et al., 2011 | Long-Evans rats | BPA | Gestational (gestational day 7 to PND 14) and lactational exposure of F ₁ offspring to 50 µg/kg bw/day through daily maternal oral administration. | <ul style="list-style-type: none"> • Perinatally BPA-exposed F₁ males exhibited sexual behavioral deficits as adults. • No disruptions were evident in the sexual behavioral of perinatally BPA exposed F₁ females. |
| Monje et al., 2009 | Inbred Wistar-derived rats | BPA | Neonatal exposure (PND 1–7) of F ₁ pups to 50 µg/kg bw/day or 20,000 µg/kg bw/day via daily subcutaneous injections. | <ul style="list-style-type: none"> • Neonatal BPA exposed F₁ females had later reductions in proceptive behaviors. • Behaviors of neonatal BPA exposed F₁ males were not assessed. |
| Panzica et al., 2005 | Male Japanese quail (<i>Coturnix japonica</i>) | BPA Estradiol benzoate (EB) DES Genistein | 50, 100, and 200 µg BPA per egg. 10 or 25 µg EB per egg. 700 ng DES per egg. 1000 µg genistein per egg. | <ul style="list-style-type: none"> • <i>In ovo</i> BPA exposure did not affect F₁ male copulatory behaviors. • <i>In ovo</i> EB, DES, and genistein abolished copulatory behaviors of pubertal F₁ males. • F₁ females were not assessed in this study. |

(Continued)

Table 1 | Continued

| Publication(s) | Animal model/human cohort population | EDC(s) tested or correlation analysis performed | Dosing regimen/method to measure BPA concentrations | Major findings |
|--|---|---|---|--|
| Wibe et al., 2002; Kaplan et al., 2013 | Threespine stickleback (<i>Gasterosteus aculeatus</i>) and Mummichog (<i>Fundulus heteroclitus</i>) | Benzyl Butyl Phthalate (BBP) | Adult F ₀ exposure to 100 µg/L in the water (0.32 µM) daily for 26 days (threespine stickleback) or 4 weeks (mummichog). | <ul style="list-style-type: none"> • BPP-treated threespine stickleback fish aggregated in a single shoal that remained at the bottom of the aquarium. • Mummichog exposed fish preferred to shoal with smaller size fish. • Potential sex differences were not assessed in this study. |
| Betz et al., 2013 | Sprague-Dawley male rats | BPP | Five to six week old F ₀ males were provided 5000 and 10,000 mg/L in the drinking water (0.16 and 0.032 µM) until they were 20–21 weeks of age (15 week duration of exposure). | <ul style="list-style-type: none"> • Juvenile to adult BPP exposed F₀ males displayed aberrant social behaviors. • F₀ females were not assessed in this study. |
| Lee et al., 2006 | Wistar-Imamichi rats | Di-n-butyl phthalate (DBP) Diisononyl phthalate (DINP) Di-2-ethylhexyl adipate (DEHA) | Perinatal exposure of F ₁ offspring through the maternal diet from gestational day 15 to weaning (PND 21) to: DBP- 20, 200, 2000, and 10,000 mg/kg bw/day DINP- 40, 400, 4000, and 20,000 mg/kg bw/day DEHA- 480, 2400, and 12,000 mg/kg bw/day | <ul style="list-style-type: none"> • DBP, DINP, and DEHA at several doses reduced copulatory behavior in perinatally exposed F₁ males. • All doses of DBP, DINP, and DEHA decreased the lordosis quotient in perinatally exposed F₁ females. |
| SOCIAL BEHAVIORS: HUMAN EPIDEMIOLOGICAL STUDIES | | | | |
| Perera et al., 2012 | 87 boys and 111 girls spanning 3–5 years of age | Maternal urinary BPA concentrations | Median maternal urinary concentrations at 34 weeks gestation: 1.8 µg/L (7.9 nM) Median child urinary concentrations at 3–4 years of age: 3.5 µg/L (15.3 nM) | <ul style="list-style-type: none"> • Linkage of prenatal BPA concentrations and increased emotionally reactive and aggressive behaviors in boys. • Opposite effect in girls with increased exposure to BPA correlating with decreased anxiety and aggressive behaviors. |
| Evans et al., 2014 | 77 boys and 76 girls spanning 6–10 years of age | Maternal urinary BPA concentrations | Median maternal urinary BPA from 10 to 39 weeks gestation with mean gestational age at collection = 26.6 weeks: 1.1 µg/L (4.8 nM) | <ul style="list-style-type: none"> • Increased maternal urinary BPA concentrations linked with greater externalizing and aggressive behaviors in boys. |
| Braun et al., 2009 | 249 mothers and their 2-year old children | Maternal urinary BPA concentrations | Median maternal urinary concentrations: 1.3–1.8 µg/L (5.6–7.9 nM), as measured at 16 and 26 weeks gestation and at birth | <ul style="list-style-type: none"> • Linkage of prenatal BPA concentrations (as determined by maternal urinary concentrations during pregnancy) and externalizing behaviors for girls but not boys. |
| Miodovnik et al., 2011 | 404 mothers and their 7–9 years of age children | Maternal urinary BPA and 10 individual phthalate metabolite concentrations | Maternal urine was collected between 25 and 40 weeks gestation (mean = 31.2 weeks). Median BPA concentration: 1.3 µg/L (5.6 nM) Median phthalate concentrations ranged from 1.6 to 430 ng/ml | <ul style="list-style-type: none"> • Linkage with phthalate exposure and later childhood social impairments, including social cognition, communication, and awareness. • No associations observed between BPA exposure and these behavioral disturbances. |

(Continued)

Table 1 | Continued

| Publication(s) | Animal model/human cohort population | EDC(s) tested or correlation analysis performed | Dosing regimen/method to measure BPA concentrations | Major findings |
|-----------------------|--|--|---|--|
| Lien et al., 2014 | 122 mothers and their 8 year old children | Maternal urinary phthalate concentrations (7 different forms) | Maternal urine was collected in the third trimester of gestation. Geometric mean range of urinary phthalate metabolite concentrations: 26.8–109.1 µg/g creatinine | <ul style="list-style-type: none"> • Positive correlation with perinatal exposure to phthalates and delinquent and aggressive behavioral scores. |
| Kobrosly et al., 2014 | 153 mothers and their 6–10 year old children | Maternal urinary phthalate concentrations (monoisobutyl phthalate, MiBP, and monobenzyl phthalate, MBzP) | Maternal urine was collected between 10 and 39 weeks gestation (mean = 26.6 weeks) Median of MiBP = 1.0 ng/ml (4.5 pM) Median of MBzP = 3.4 ng/ml (13.3 pM) | <ul style="list-style-type: none"> • Boys of mothers with higher urinary monoisobutyl phthalate concentrations were more likely to show higher scores for inattention, rule-breaking behavior, aggression, and conduct problems. • Increased concentration of monobenzyl phthalate was correlated with conduct problems in boys but reduced anxiety scores in girls. |

exposed for 1–4 weeks to 11 ng/L water (36.7 pM) of 17 α -ethinyl estradiol (EE2, estrogen in birth control pills) showed suppressed nest building activity, along with decreased courtship behaviors. These combined deficiencies thus likely effect successful fry rearing by exposed males. Future work is needed in monogamous and biparental rodent and other animal models to determine how developmental exposure to EDC affects paternal care and parenting providing by his partner.

EVIDENCE THAT ENDOCRINE DISRUPTORS AFFECT SOCIAL BEHAVIORS

Extensive data from retrospective human studies and animal models links perinatal exposure to EDCs and later social deficits (Table 1). The evidence in animal models will first be considered. Social behaviors to be considered include any that involve an interaction between difference members of the same species, such as various forms of communication, mate choice, pair bonding, social inquisitiveness and recognition, play behavior, social grooming, copulation, and aggression.

ANIMAL MODELS

Animal communication assumes various forms besides direct vocalizations. In California mice (*Peromyscus californicus*), males use territorial marking to communicate and protect their home range and mate from intruders. Males pericoceptionally through perinatally exposed (2 weeks prior to breeding of the dam through weaning at PND 30) to BPA (50 mg/kg feed weight in the maternal diet) show reduction of this behavior when a control male is present in the testing arena (Williams et al., 2013). BPA exposure (1280 µg/L water, 5.6 µM for 14 days) of native black-tail shiner fish (*Cyprinella venusta*) and introduced red shiner fish (*C. venusta*) disrupts normal visual communication signals

and abolishes the species-dependent sexually selected behavioral traits with the net potential for hybridization to occur between these two otherwise isolated species (Ward and Blum, 2012). Sprague-Dawley female rats prenatally exposed from gestational days 16 to 18 via daily intraperitoneal injection to the PCB mixture Aroclor (A) 1221 (100 µg/kg, 1000 µg/kg, and 10,000 µg/kg bw/day) have later vocalization deficits, as well as changes in mating patterns, movement and likelihood to mate (Steinberg et al., 2007).

Mate choice and pair-bonding formation are vulnerable to endocrine disruption. In mate choice trials, control and BPA-exposed female deer mice (*P. maniculatus bairdii*) selectively reject males developmentally exposed to BPA (50 mg/kg feed weight through the maternal diet) (Jasarevic et al., 2011). Likewise, female F₃ Sprague-Dawley rats prefer F₃ males whose ancestors were not prenatally exposed to the anti-androgenic EDC, vinclozolin (100 mg/kg bw/day from gestational days 8 to 14 via daily intraperitoneal injection) (Crews et al., 2007). Female sand gobies favor mating with control males, as opposed to those exposed for 7–24 days to EE2 (4 ng/L water, 13.3 nM) (Saaristo et al., 2009). Pair-bonded monogamous Mongolian gerbil (*Meriones unguiculatus*) females treated for a 3 week duration (from pairing to day 21 of cohabitation) with daily oral administration of either BPA (2 or 20 µg/kg bw/day) or EE2 (0.04 µg/kg bw/day) exhibit heightened investigation of their partner and less exploration than untreated controls (Razzoli et al., 2005).

Introduction of new animals into the habitat results in social inquisitiveness and in time recognition of the new member with reduced bouts investigating this individual, i.e. habituation. Developmental and transgenerational exposure to BPA (5000 µg/kg feed weight through the F₀ maternal diet 7–10 days prior to being paired with a breeder male and for 2 weeks thereafter) prolonged the duration of time juvenile F₁ and F₃

C57BL/6 mice, respectively, spend investigating a novel animal (Wolstenholme et al., 2013). Further, F₃ females, whose ancestors were exposed to BPA, persisted in investigating novel females longer than controls, suggestive of impaired dishabituation.

Exposure to BPA may affect other socio-sexual behaviors, including play, social investigation, copulation, and aggression. Decrease amount of play with males and social grooming were evident in female Sprague-Dawley rats perinatally exposed to BPA (4000 µg/kg bw/day to the dams via daily oral administration from mating through weaning at PND 21), which indicates that this chemical can defeminize select female social behaviors (Porrini et al., 2005). Sprague-Dawley rats exposed perinatally to BPA (40 mg/kg bw/day from conception to weaning or 400 mg/kg/day from gestational day 14 to postnatal day 6 via daily maternal oral dosing) demonstrated masculinization of female social behaviors with such females selectively preferring to play with and engage in socio-sexual exploration of other females (Dessi-Fulgheri et al., 2002). Another study though with C57Bl6J mice suggests that gestational exposure to 1250 µg BPA/kg feed weight increased play solicitations and approaches by both sexes (Wolstenholme et al., 2011b).

Sexual behaviors are also vulnerable to the early effects of BPA. Male LE rats, who received a “low dose” BPA (50 µg/kg bw/day through maternal oral administration spanning gestation and lactation (gestational day 7 through PND 14), were sexually incompetent as adults, but no deficits in female sexual behavior were noted in this study (Jones et al., 2011). In contrast, another report found that neonatal exposure of inbred Wistar-derived female rats to BPA (50 µg/kg bw/day or 20,000 µg/kg bw/day via daily subcutaneous injections to the pups) had later reductions in proceptive behaviors (Monje et al., 2009). While BPA exposure (50, 100, and 200 µg per egg) did overtly effect male Japanese quail (*Coturnix japonica*) copulatory behaviors, *in ovo* treatment with other estrogenic chemicals, including estradiol benzoate (10 or 25 µg per egg), DES (700 ng per egg), and the soy phytoestrogen, genistein (1000 µg per egg), abolished this behavior in pubertal males (Panzica et al., 2005).

Current data demonstrate that phthalates can also affect varying social behaviors in rodents and fish. Benzyl butyl phthalate (BBP) exposure through the water (100 µg/L, 0.32 µM) altered the shoaling behaviors (collection of fish for social purposes) in threespine stickleback (*Gasterosteus aculeatus*), who were treated each day for 26 days, and mummichog, a small killifish, (*Fundulus heteroclitus*), who were treated daily for 4 weeks (Wibe et al., 2002; Kaplan et al., 2013). BPP-treated threespine stickleback fish tended to aggregate in a single shoal that remained at the bottom of the test aquarium; whereas mummichog exposed fish preferred to shoal with smaller size fish. Male Sprague-Dawley rats exposed from adolescence to adulthood (5–6 weeks of age to 20–21 weeks of age) to BPP through the drinking water (5000 µg/L and 10,000 µg/L or 0.016 and 0.032 µM, respectively) displayed aberrant social behaviors (Betz et al., 2013). Male and female Wistar-Imamichi rats developmentally exposed through the maternal diet from gestational day 15 through weaning (PND 21) to various phthalate chemicals (di-n-butyl phthalate, DBP-20, 200, 2000, and 10,000 mg/kg bw/day; diisononyl phthalate, DINP-40, 400, 4000, and 20,000 mg/kg bw/day; di-2-ethylhexyl

adipate, DEHA-480, 2400, and 12,000 mg/kg bw/day) exhibited sexual behavioral deficits (Lee et al., 2006). Copulatory behavior was diminished in male rats exposed to the varying doses of these chemicals. Similarly, the lordosis quotient was reduced in all treatment group females.

HUMAN EPIDEMIOLOGICAL STUDIES

Associations between early BPA exposure and social behavioral disruptions in children are generally based on urinary BPA concentrations in the mother or child (Table 1). An African-American and Dominican women and their children population cohort that included 87 boys and 111 girls spanning 3–5 years of age showed higher exposure of BPA correlated with increased aggressive behaviors in boys but decreased aggression in girls (Perera et al., 2012). A more recent study with 153 six to ten year old children (77 boys and 76 girls) also demonstrated similar sex differences in vulnerability with increased maternal urinary BPA concentrations linked with greater externalizing and aggressive behaviors in boys (Evans et al., 2014). However, a study examining 249 mothers and their 2-year old children reported elevated prenatal BPA exposure associated with greater externalizing (aggression and hyperactivity) in girls but not boys (Braun et al., 2009).

Perinatal exposure to phthalates is also correlated with social behavioral disturbances. A study population of 404 mothers and their 7–9 years of age children pairs linked early contact to this chemical and later childhood social impairments, including social cognition, communication, and awareness (Miodovnik et al., 2011). This study however did not find any association between BPA exposure and effects on the examined social behaviors or sex-dependent differences. Examination of 122 mother-8 year old child pairs from Taiwan showed positive correlation of delinquent and aggressive behavioral scores and early exposure to different phthalate chemicals, as evidenced by maternal urinary concentrations (Lien et al., 2014). However, this study did not examine for potential sex-dependent differences. Another study with 153 mothers and their 6–10 year old children indicates that prenatal phthalate exposure is associated with later sex-dependent behavioral disturbances (Kobrosly et al., 2014). Boys of mothers with higher urinary monoisobutyl phthalate concentrations were more likely to show higher scores for inattention, rule-breaking behavior, aggression, and conduct problems; whereas increased concentration of monobenzyl phthalate was correlated with conduct problems in boys but reduced anxiety scores in girls.

MECHANISMS BY WHICH ENDOCRINE DISRUPTORS MAY AFFECT PARENTAL AND SOCIAL BEHAVIORS

DISRUPTION OF STEROID HORMONE PRODUCTION OR AT THE STEROID RECEPTOR LEVEL

As detailed above, full elaboration of many social and parental behaviors is dependent on the organizational and activational effects of testosterone and estrogen (Phoenix et al., 1959; Arnold and Breedlove, 1985; Morris et al., 2004). Figure 1 illustrates the primary factors and enzymes required for normal testosterone and estrogen production. Both BPA and phthalates can affect steroidogenesis at several points in the pathway for males and females. Steroidogenic acute regulatory protein (StAR, STARD1)

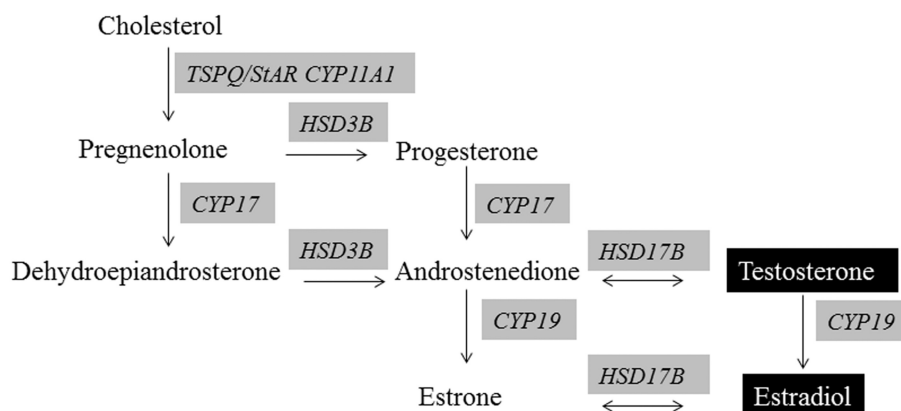


FIGURE 1 | Steroidogenesis of androgens and estrogens. Evidence exists that all of the shaded enzymes required in the synthesis of these hormones are altered by BPA and/or phthalate exposure.

is an essential mitochondrial protein for transporting cholesterol into the cell and is thus considered the rate-limiting step for steroid hormone production. BPA suppresses *StAR* mRNA expression in male and female gonads of rodents and fish (D'Cruz et al., 2012; Horstman et al., 2012; Liu et al., 2012; Peretz and Flaws, 2013; Savchuk et al., 2013). Expression of gonadal steroidogenic enzymes, including *Cyp11*, *Hsd3b*, *Hsd17b*, *Cyp17*, *Cyp19* is also generally inhibited by BPA exposure (D'Cruz et al., 2012; Liu et al., 2012; Nanjappa et al., 2012; Peretz and Flaws, 2013; Savchuk et al., 2013).

Most animal model studies report BPA exposure decreases production of testosterone (T) and estrogen (E2) by male and female gonads (Akingbemi et al., 2004b; Peretz et al., 2011; D'Cruz et al., 2012; Nanjappa et al., 2012). However, a study with isolated porcine ovarian follicles suggests potential dose-dependent effects with lower dose BPA concentrations (0.1 mM) elevating E2; whereas large doses (1 and 10 mM) suppressing E2 levels (Grasselli et al., 2010). *In ovo* BPA treatment (0.01 or 1.4 ppm) of *Caiman latirostris* eggs elevated E2 and lowered T concentrations and concomitantly reversed the normal temperature sex determination mechanisms with a predominance of females occurring in these groups (Stoker et al., 2008). In girls with precocious puberty, elevated urinary BPA concentrations were associated with elevated T, E2, and pregnenolone levels (Lee et al., 2014). Phthalate exposure is also associated with decreased *StAR* and steroidogenic enzyme expression and steroid hormone production (T and E2) by the male gonad (Akingbemi et al., 2001, 2004a; Svechnikov et al., 2008; Botelho et al., 2009; Chauvigne et al., 2011; Desdoits-Lethimonier et al., 2012; Saillenfait et al., 2013; Beverly et al., 2014), as well as circulating concentrations of T and E2 in men (Meeker et al., 2009). In contrast, one report identifying disrupted social interactions in phthalate-treated rats indicated that this chemical increased E2 concentrations (Betz et al., 2013).

BPA and phthalates may also disrupt normal organizational/activational steroidogenic effects by altering the expression of the cognate receptors, ESRs and AR, in the neural regions governing social and parental behaviors, namely the hypothalamus.

In this region, developmental exposure to BPA has been shown to alter the expression of ESR1 and ESR2 in rodent and ovine models, although the directionality is seemingly dependent upon sub-region, dose, time of assessment, and possibly species (Ramos et al., 2003; Ceccarelli et al., 2007; Monje et al., 2007; Mahoney and Padmanabhan, 2010; Cao et al., 2012, 2013, 2014; Patisaul et al., 2012; Kundakovic et al., 2013). Scant information exists on how phthalates may affect neural expression of ESRs and AR. The aforementioned rat phthalate study above also showed increased ESR1 expression in the amygdala of rats treated with 5 mg/L (0.16 μ M) BBP (Betz et al., 2013). Combined treatment of 4 wk old rats with BPA (285.4 mg/kg) and DBP (285.4 mg/kg) in the feed increased AR expression in the brain (Zhang et al., 2013), which might render these animals more sensitive to endogenous and exogenous androgens.

DISRUPTION OF THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS

BPA, phthalates, and other EDC exposure may disturb parental and social behaviors through disruptions in the classic stress-associated adrenocortical axis. One rat study showed that early BPA exposure (oral administration of 40 μ g/kg bw/day to the dam throughout gestation and lactation) led to sex dependent effects on circulating corticosterone concentrations (elevated in BPA-exposed females) and neural (hippocampal expression) of *Gr* (lower expression in BPA-exposed males) (Poimenova et al., 2010). Further, BPA-treated female rats developmentally exposed to this same dosing regimen exhibited increased basal corticosterone and suppressed hypothalamic *Gr* expression (Panagiotidou et al., 2014).

Another study in rats where dams were subcutaneously injected with 2 μ g BPA/kg bw/day from gestational day 10 to lactational day 7 showed sex-dependent alterations in the adreno-cortical axis (Chen et al., 2014). BPA-exposed male rats had increased basal concentrations of serum corticosterone and adrenocorticotropin (ACTH) and corticotropin-releasing hormone (*Crh*) mRNA expression, but these changes were not evident in exposed females. Following subsequent exposure to a mild

stressor, corticosterone and ACTH concentrations increased further in BPA males; whereas, these hormones decreased in BPA females. In non-stressed animals, *Gr* mRNA was increased in the hippocampus and hypothalamic paraventricular nucleus of BPA females. In contrast, these transcripts decreased in BPA males. The collective results suggest that BPA exposure might affect the HPA axis in males and females but the specific effects are sex-dependent.

Phthalates also appear to disrupt the HPA axis. Oral exposure to DEHP (500 mg/kg bw/day for 4 days) increased concentrations of ACTH and corticosterone in treated male rats at 20 and 40 days of age; whereas no effects were observed in adult (60 days of age) males (Supornsilchai et al., 2007). Isolated adrenocortical cells from the 20 and 40 day old treated rats were more sensitized to the effects of ACTH, dibutyl cAMP, and 22R-hydroxylase, as evidenced by increased production of corticosterone and ACTH treatment stimulated greater transport of endogenous cholesterol into the mitochondria.

DISRUPTION OF NEUROPEPTIDE/PROTEIN HORMONES AND THEIR COGNATE RECEPTORS

In the mouse and rat, central oxytocin receptors (OXTR) are obligatory for the expression of maternal behavior, and variation in OXTR levels in the medial preoptic area of the hypothalamus (MPOA) are functionally linked to differences in degree of maternal care (Champagne et al., 2006; Weaver, 2007). California mice fathers are known to exhibit increased oxytocin immunoreactivity compared to non-paternal males (Lambert et al., 2011). Increased signaling through the OXTR is correlated with high degree of maternal behavior, such as pup licking and grooming, while *ESR1* is an important regulator of OXTR expression (Champagne et al., 2006). Prolactin, oxytocin, and vasopressin have been implicated in paternal care in California mice, monogamous prairie voles (*Microtus ochrogaster*), and humans (Gubernick and Nelson, 1989; Parker and Lee, 2001; Bales et al., 2004; Wynne-Edwards and Timonin, 2007; Gordon et al., 2010). Social behaviors are also dependent upon oxytocin, vasopressin, and protein binding to their cognate receptors in the brain (Carlson et al., 2006; Benarroch, 2013; Walker and McGlone, 2013; Wudarczyk et al., 2013; Babb et al., 2014; Lieberwirth and Wang, 2014).

Brains from 18.5 days post-coitus (dpc) mice exposed to BPA express less oxytocin, OXTR, and vasopressin than control males (Wolstenholme et al., 2011a, 2012). Reductions in brain expression for vasopressin persists in transgenerationally BPA exposed F₄ males and females, and oxytocin is decreased in F₄ males (Wolstenholme et al., 2012). Another study, however, suggests that in the paraventricular nucleus (PVN), oxytocin-immunoreactive neurons increase in neonatally exposed BPA female Long Evans rats (Adewale et al., 2011), but non-pregnant, non-lactating females were examined. A recent study with juvenile prairie voles showed neonatal exposure to BPA increased AVP-immunoreactive neurons in the anterior PVN but decreased OT-immunoreactive neurons in this same region (Sullivan et al., 2014). *In vivo* and *in vitro* studies suggest that BPA increases PRL levels in male and female rats (Steinmetz et al., 1997; Goloubkova et al., 2000; Ramos et al., 2003; Delclos et al., 2014). Likewise, phthalate exposure is associated with elevated prolactin production in rats of both sexes and men (Lee et al., 2004; Li et al.,

2011). No previous study has assessed the effects of BPA or phthalate-exposure on PRL in parenting males and females.

EPIMUTATIONS

Epimutations are the most plausible mechanisms by which early exposure to BPA, phthalates, and other EDCs may lead to later neurobehavioral disturbances. Such changes may include alteration in DNA methylation, histone proteins, non-coding RNA, or chromatin arrangement. It is now apparent from a variety of animal model studies that BPA, phthalates, and other EDCs can lead to DNA methylation and corresponding gene expression changes in a variety of tissues, including the brain (Yaoi et al., 2008; Jang et al., 2012; Tang et al., 2012; Kundakovic et al., 2014; Zhao et al., 2014; Martinez-Arguelles and Papadopoulos, 2015).

Only two studies to date have shown BPA-induced DNA methylation changes in the brain. Developmental exposure to BPA alters the DNA methylation promoter state in many genes of the mouse forebrain (Yaoi et al., 2008). Persistent DNA methylation changes are evident in one of the promoters of *Bdnf* in the hippocampus and cord blood in female and male mice subjected to prenatal exposure to BPA with hypomethylation apparent in females but hypomethylation observed in males (Kundakovic et al., 2014). DNA methyl transferase (DNMT) and methyl CPG binding proteins (MECP) guide global DNA methylation changes.

BPA may simultaneously effect DNA methylation and histone protein modifications. For instance, exposure to BPA suppresses rat brain cortical expression of the ion transporter (*Kcc2*) through both DNA methylation via MECP2 and histone protein (H3K9) binding to this gene (Yeo et al., 2013). Phthalates can also induce histone protein modifications in neuronal cells, as evidenced by phthalate-induced Sp3 suppression associated with deacetylation (via HDAC4) and ensuing polyubiquitination in neuroblastoma cells (Guida et al., 2014).

The expression of *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Mecp2* is altered in various brain regions, including basolateral amygdala, cortex, and hypothalamus, in BPA-treated rodent models (Kundakovic et al., 2013; Warita et al., 2013; Zhou et al., 2013). The histone protein modification enzyme (HDAC2) is up-regulated in the hippocampus of adult males exposed to BPA (Zhang et al., 2014a). The histone methyltransferase enzyme (EZH2) and histone H3 trimethylation is upregulated in the mammary gland of mice exposed to BPA *in utero* and MCF-7 cells treated with this chemical (Doherty et al., 2010).

While no study has determined whether BPA exposure affects non-coding (nc) RNAs (including microRNAs, miRNAs) in the brain, there is evidence that this chemical can affect expression of these biomolecules in other cells, including MCF7 (Tilghman et al., 2012), ovarian (Veiga-Lopez et al., 2013), and placental cells (Avisar-Whiting et al., 2010). No study to date has assessed whether phthalate exposure affects ncRNAs in the brain or other regions.

CONCLUSIONS

Strong evidence exists that parental and social behaviors in a wide variety of species, including by translation humans, are vulnerable to perinatal exposure to EDCs, including BPA and phthalates. These effects are likely multifactorial in origin, but

the net result is presumably that these chemicals disrupt normal organizational and activational programming of the brain. EDC-induced disruptions on neural programming may occur as a result of alterations in fetal or adult steroid hormone production, steroid receptor expression in the brain regions governing these traits, neuropeptide/protein hormones and their cognate receptors, and/or through epimutations. It remains to be determined though whether BPA, generally considered a “*weak estrogen*,” results in similar mechanistic disruptions as phthalates with their predominantly anti-androgenic effects. Both chemicals can potentially disrupt all of the above pathways. Further, it is now apparent that both chemicals may lead to pleiotropic disturbances through steroidogenic and non-steroidogenic pathways (Leon-Olea et al., 2014).

EDC-induced parental and social behavioral deficits could affect the general livelihood, social well-being, ability to attract mate(s), reproduction, and likelihood of successfully rearing young. Thus, early exposure of animal and humans to these widely prevalent chemicals may lead to insidious behavioral effects. Moreover, there is ample evidence in animal models that EDCs (and humans in the case of DES) can induce transgenerational effects (Newbold et al., 2000, 2006; Klip et al., 2002; Titus-Ernstoff et al., 2006; Wolstenholme et al., 2012, 2013; Doyle et al., 2013; Manikkam et al., 2013; Schneider et al., 2013; Zhang et al., 2014b). Therefore, these chemicals might already be exhuming a toll on the social lives of unborn generations. While a call to action to legislate further these chemicals seems a reasonable course of action based on the existing data and precautionary principle, identifying the underpinning mechanisms leading to these behavioral disturbances might provide the incentive for policymakers to act. Long-term studies examining for linkages in parental deficiencies and transgenerational effects in human populations exposed to these EDCs may regrettably be needed before the production of such chemicals is minimized or outright banned.

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A multi-oscillatory circadian system times female reproduction

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Rhythms in female reproduction are critical to insure that timing of ovulation coincides with oocyte maturation and optimal sexual arousal. This fine tuning of female reproduction involves both the estradiol feedback as an indicator of oocyte maturation, and the master circadian clock of the suprachiasmatic nuclei (SCN) as an indicator of the time of the day. Herein, we are providing an overview of the state of knowledge regarding the differential inhibitory and stimulatory effects of estradiol at different stages of the reproductive axis, and the mechanisms through which the two main neurotransmitters of the SCN, arginine vasopressin, and vasoactive intestinal peptide, convey daily time cues to the reproductive axis. In addition, we will report the most recent findings on the putative functions of peripheral clocks located throughout the reproductive axis [kisspeptin (Kp) neurons, gonadotropin-releasing hormone neurons, gonadotropic cells, the ovary, and the uterus]. This review will point to the critical position of the Kp neurons of the antero-ventral periventricular nucleus, which integrate both the stimulatory estradiol signal, and the daily arginine vasopressinergic signal, while displaying a circadian clock. Finally, given the critical role of the light/dark cycle in the synchronization of female reproduction, we will discuss the impact of circadian disruptions observed during shift-work conditions on female reproductive performance and fertility in both animal model and humans.

Keywords: female reproduction, circadian clock, suprachiasmatic nuclei, kisspeptin, GnRH, LH, estradiol, shift-work

Introduction

Ovulation in female mammals is a complex process, which is exquisitely regulated by a number of environmental (time of day, time of year, food resources, and stress level) and internal (development stage, hormonal milieu, and metabolic rate) factors. Indeed, female reproduction is a long-term, demanding process and therefore, it is important that a limiting critical status is reached to ensure successful reproductive outcome. In adult females where all these criteria are attained, there are still two important cues that time ovulation: the circulating level of gonadal hormones, specifically estradiol, which is an indicator of oocyte maturation, and the time of day arising from biological clocks. This dual regulation ensures that the timing of ovulation coincides with the period of maximal activity and sexual motivation. Most mechanistic studies aimed at understanding this subtle timing of ovulation have been performed in laboratory rodents, but ovulation in humans is also gated by similar hormonal and circadian inputs. Hence, this review will not only focus on the mechanisms regulating the timing of reproduction in female rodents, but will also discuss human female fertility, including the desynchronization associated with modern life styles (shift work, jet lag, and sleep alteration).

Female Reproduction is Rhythmic

Reproductive activity in female mammals displays a regular cycle (menstrual cycles in women, estrous cycles in rodents) driven by a complex interaction of the circadian system, hypothalamic neuropeptides, gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH), both secreted by the pituitary gonadotroph cells], and sex steroid hormones produced by the ovaries. The final output of this regulatory process is to combine the production of a mature oocyte (ovulation) with a receptive reproductive tract, which will ensure the embryo's development.

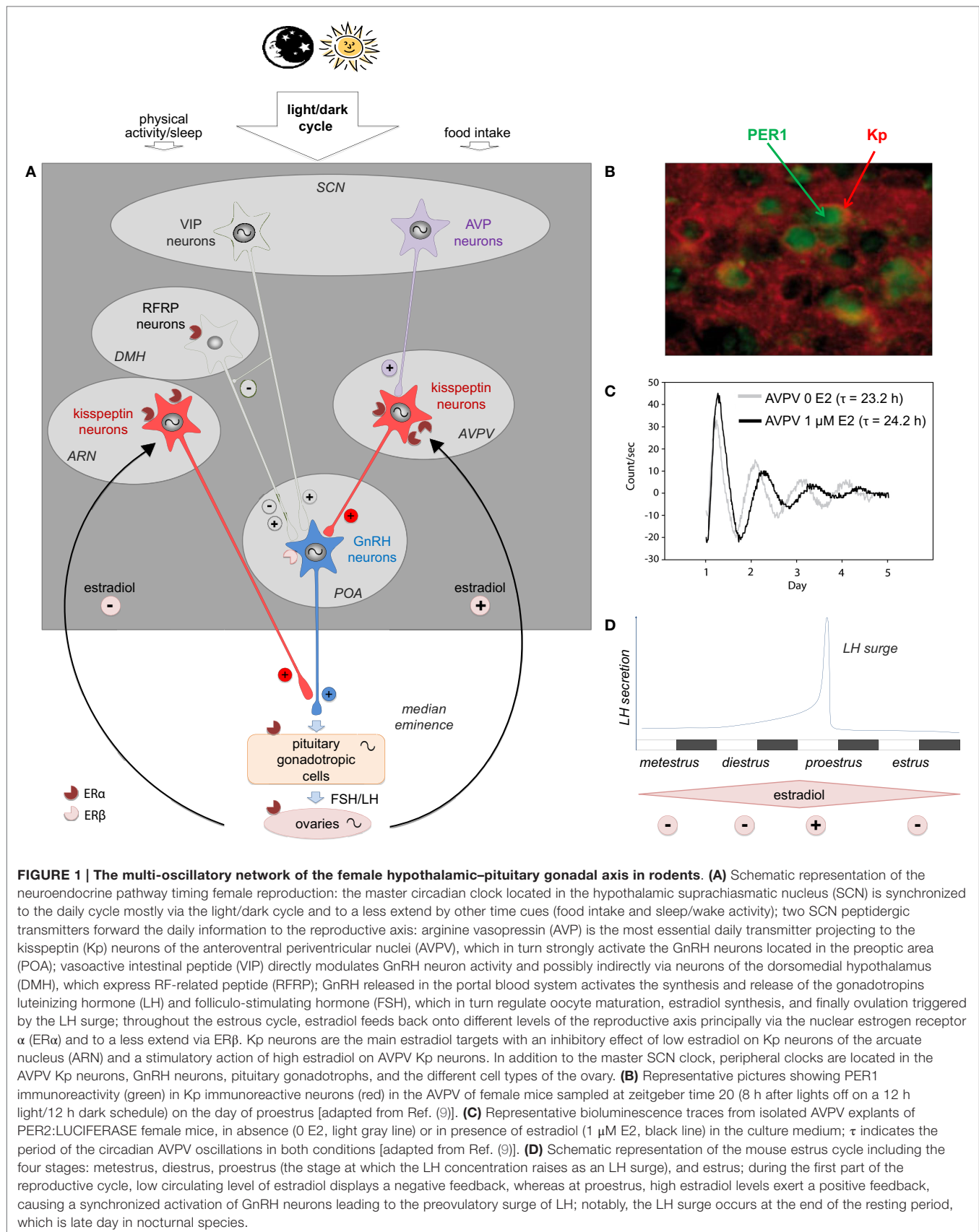
During the first part of the reproductive cycle (follicular phase in women; metestrus–diestrus in rodents), gonadotrophs produce more FSH than LH. This relative FSH preponderance contributes to the recruitment and development of ovarian follicles. FSH promotes follicular growth leading to a progressive increase of the sex steroid hormone, estradiol, and increases LH receptor expression in granulosa cells (1). During this early phase, LH pulses occur with a high frequency (period of 1–2 h in women, 20 min in rodents) and uniform amplitude, and the pulse frequency tends to increase toward the end of the phase. The second part of the reproductive cycle (luteal phase in women; proestrus–estrus in rodents) begins with a marked and transient secretion of LH (**Figures 1A,D**). The LH surge has three functions: (1) induction of ovulation of mature follicles, (2) resumption of oocyte meiosis, and (3) arrest of granulosa cell proliferation and luteum induction. After the LH surge, ovulation generally follows a few hours later in rodents, and 24–48 h in women. The preovulatory LH surge takes place approximately every 4–5 days in rodents and every 28 days in women and its occurrence depends on high circulating estradiol levels (2, 3). Additionally, the LH surge requires a daily signal since it arises at a very specific time of day, usually at the end of the resting period, thus in the late afternoon in nocturnal rodents and the end of the night/early morning in the diurnal rodent *Arvicanthis* (4) and in humans (5–7). Indeed, in 80% of women, the LH surge occurs around 8 a.m. At the end of the reproductive cycle, LH pulse frequency decreases significantly down to a pulse interval of 2–6 h with variable amplitude (8).

The secretion of both LH and FSH is under the control of a hypothalamic neurohormone, gonadotropin-releasing hormone (GnRH), which is synthesized in neurons scattered throughout the preoptic area (POA) and the organum vasculosum laminae terminalis. These neurons project to the median eminence where they release GnRH in the portal circulation in a pulsatile manner. GnRH activates specific receptors located on pituitary gonadotrophs inducing the synthesis and release of LH and FSH. GnRH is essential for reproduction as mutations in the gene coding for GnRH (10) and GnRH receptor (11) are proposed to be responsible for idiopathic hypogonadotropic hypogonadism (IHH), characterized by delayed puberty and infertility (11). The pulsatility of GnRH release is critical to induce proper gonadotropin secretion, and there is a tight correlation between GnRH and LH pulsilities. Pulsatile administration of exogenous GnRH (one pulse per hour) is capable of restoring the preovulatory surge, ovulation, and normal menstrual cycles in patients suffering from Kallmann syndrome (12, 13). In contrast, continuous GnRH administration

induces a reversible blockage of the pituitary gonadotroph cells' secretion (14).

Various (neuro)transmitters had been proposed to regulate GnRH neuronal activity until the neuropeptide kisspeptin (Kp) was discovered as a potent activator of GnRH release. In 1996, the *Kiss1* gene was discovered and reported to encode a peptide called metastin, because of its anti-metastatic property on malignant melanoma cells (15). However, the receptor of this peptide, GPR54, was later found to play a critical role in reproductive physiology when two groups reported that mutation of the *GPR54* receptor results in IHH in humans, with an identical phenotype observed in mice with a targeted deletion in this receptor (16, 17). The *Kiss1* gene was shown to encode a family of Kps from an initial 145 amino acid propeptide, Kp-145, which is cleaved into peptides of different sizes from Kp-54 (previously named metastin) to Kp-10. The discovery of Kp's role in reproductive function has been a milestone in the field of reproductive biology, and numerous studies now indicate that Kps are critical regulators of sexual differentiation and maturation as well as of normal adult reproductive functioning across mammalian species, including humans (18). Kp neurons are localized within two hypothalamic areas, in the arcuate nucleus (ARN) and the rostral periventricular nucleus of the third ventricle, also called anteroventral periventricular nucleus (AVPV), or the preoptic area (according to species). They send projections mainly to the GnRH neuron cell bodies (AVPV Kp neurons) and nerve terminals [ARN Kp neurons (19–22)] (**Figure 1A**). The AVPV presents a marked sexual dimorphism, with more Kp neurons in females as compared to males (20, 23). The AVPV Kp neurons are the main drivers of the preovulatory GnRH/LH surge (24). In contrast, the ARN Kp neurons are not sexually dimorphic (20, 23). The Kp receptor, Kiss1R (formerly GPR54), is highly expressed in GnRH neurons but also in other brain areas (25, 26) and in most endocrine tissues like the pituitary gland, ovary, and placenta (27). Kp has a very potent stimulatory action on GnRH release and, therefore, gonadotropin secretion in all mammalian species investigated so far (18, 19, 28, 29). Central injection of doses as low as 0.1–1 pmol Kp10 is indeed sufficient to evoke robust LH secretion in rats and monkeys (28, 30). Kp injections must be short and at least 2 h apart to induce the LH peak since the repeated administration of Kp induces Kiss1R desensitization (31, 32). Notably, Kp release in the stalk-median eminence is pulsatile (33), and pulsatile Kp drives LH secretion in juvenile monkeys (34). A recent study reported that pulsatile administration of Kp was able to evoke dramatic synchronous activation of *GnRH* gene transcription with robust stimulation of GnRH secretion in murine-cultured hypothalamic explants (35). The preeminent phenotypes of impaired reproduction (abnormal sexual maturation, small uterus, ovaries without mature follicles, no estrous cycles) often arise from mutations in *Kiss1* (36, 37) and *Kiss1R* (16, 38, 39), which suggest that the Kiss1/Kiss1R complex is essential for the central regulation of the gonadotropic axis.

Other classical neurotransmitters and neuropeptides have been reported to regulate GnRH neuron activity albeit not to the same extent as Kp. GABA and glutamate fibers are found close to GnRH perikarya in the POA and axons in the median eminence. Both neurotransmitters have been shown to play a role in the regulation of GnRH release. Glutamate stimulates *Gnrh* gene



expression and GnRH release during the LH surge, whereas a glutamate antagonist blocks *Gnrh* gene expression and the LH surge when administered in the morning (40–42). Administration of an AMPA agonist enhances the *in vivo* LH secretion in OVX rats only with estradiol substitution, whereas glutamate stimulates *in vitro* GnRH secretion in a estradiol-independent manner (43). The role of GABA on GnRH neuronal activity is debated since inhibitory and stimulatory effects have been observed depending on the protocols used, the presence of sex steroid treatment, the timing in the estrus cycle and the hypothalamic region studied (44, 45). Fibers containing the orexigenic neuropeptide Y contact a majority of GnRH neurons, which express neuropeptide Y receptors. This neuropeptide has been reported to exert variable effects depending on the metabolic and reproductive status of the animal, but most of the studies describe an inhibitory effect of neuropeptide Y on GnRH neurons (46–48). Recent studies indicate that another neuropeptide belonging to the same RF-amide peptide family as Kp, RFRP-3 (the mammalian homolog of avian gonadotropin-inhibitory hormone), regulates GnRH neuron activity [for review, see Ref. (49–51)]. Unlike Kp, RFRP-3 can activate or inhibit the reproductive axis according to gender, species, and environmental conditions (26, 51–53). In female rodents RFRP neuronal activity is decreased at the time of the LH surge, possibly to relieve the inhibitory effect of RFRP-3 on GnRH neurons (54). However, mice bearing a null mutation of GPR147, the RFRP-3 receptor, present only a mild reproductive phenotype (55).

Estrogenic Regulation

Estradiol produced by the ovaries exerts both positive and negative feedback upstream of the reproductive axis, modulating GnRH neuron activity and anterior pituitary gonadotroph cells. During the first part of the reproductive cycle, estradiol induces a negative feedback, whereas when estradiol concentration is the highest (at the end of the follicular phase in humans or proestrus in rodents) the feedback becomes positive, causing a synchronized activation of GnRH neurons leading to GnRH release in the hypophyseal portal blood and finally the preovulatory LH surge (56) (**Figures 1A,D**). The effect of estradiol is mediated via two types of nuclear estrogen receptors (ERs), which induce long lasting genomic action, ER α and ER β (57, 58), but it can also have a rapid action via membrane bound estradiol receptors, including the GPR30 (59, 60).

GnRH Neurons

Gonadotropin-releasing hormone neurons contain few, if any, ER α (61–63), but do express ER β (64–66) and GPR30 (67). Estradiol application to cultured primate GnRH neurons induces a rapid increase in action potential firing frequency (68) and intracellular calcium oscillations (69). Similar effects have been reported in the mouse GnRH neurons (70). This rapid effect of estradiol is proposed to be mediated via GPR30 in primates (67) and ER β in mice (70, 71). Using an *in vitro* GnRH neuronal model, the GT1-7 cells, it was reported that *Kiss1R* expression is estradiol dependent, with a Kp-induced GnRH increase only in cells treated with estradiol (72, 73). A primary effect of estradiol on GnRH neurons

has been hypothesized to upregulate expression of channel transcripts (TRPC4 channels and HCN1 channels) that orchestrate the downstream signaling of Kiss1R in GnRH neurons (74, 75). Therefore, estradiol could be a potent regulator of ion channel and receptor expression in GnRH cells, hence controlling the sensitivity of GnRH neurons to Kp. However, a recent study reported that mutant mice with a GnRH neuron-selective deletion of ER β exhibit normal cycles and negative feedback, leaving the critical role for ER β in GnRH neuron activity still an open question (76).

Kisspeptin Neurons

In contrast to GnRH, Kp neurons have a high density of ER α and are, therefore, considered as the intermediate node for the estradiol feedback on GnRH neurons (19, 20, 32, 61, 77–79) (**Figure 1A**). Interestingly, the estradiol feedback in rodents depends on the Kp neuron localization as estradiol stimulates *Kiss1* expression in the AVPV and inhibits *Kiss1* expression in the ARN (77, 80–82). In non-rodent mammals, a similar differential regulation by estradiol is also observed with a stimulatory effect in the rostral periventricular/POA and an inhibitory effect in the ARN (83, 84). The mechanism underlying these differences in the regulation of *Kiss1* expression by ER α in both structures is not yet fully understood. AVPV *Kiss1* activation requires an estrogen response element (ERE)-dependent pathway, whereas inhibition of *Kiss1* expression in the ARN involves ERE-independent mechanisms (80, 85). Recent studies have reported that estradiol additionally modulates daily activity of AVPV Kp neurons. Hence, the daily variation in c-Fos activation, *Kiss1* mRNA and peptide content observed in proestrus or in ovariectomized estradiol-treated rodents is abolished or strongly reduced in diestrus or in ovariectomized animals (9, 86–88). Furthermore, estradiol has been reported to increase the number of arginine vasopressin (AVP) synaptic contacts onto Kp neurons (89), regulate AVP_{1a} receptor expression by Kp neurons (88) and be permissive for the AVP-induced electrical activation of Kp neurons (90). Altogether these observations indicate that high circulating levels of estradiol gate the action of AVP onto AVPV Kp neurons (see Role of the Suprachiasmatic Nuclei for the role of AVPV on Kp neurons).

Circadian System

Estradiol is also suggested to influence daily functions since shifts are observed during the pubertal period, pregnancy, menopause, and throughout the reproductive cycle (91–93). In rodents, ER α and ER β are expressed in the retina, the retino-hypothalamic tract, the geniculohypothalamic tract and raphe nuclei-derived serotonergic inputs, all major inputs to the suprachiasmatic nuclei (SCN), which contain the master circadian clock (94). In mice, the second half of the proestrus night is often, but not consistently, characterized by increased motor activity compared to the remaining nights of the estrous cycle (95, 96). Furthermore, ovariectomy reduces total motor activity, and estradiol reverses this effect, while also shortening the length of the free running period and advancing the onset of wheel running activity (96). Estradiol may act directly on SCN clock gene oscillation since estradiol treatment in ovariectomized rats decreased *Cry2* mRNA levels (97) and estradiol application to SCN slices increased the spontaneous firing frequency and depolarized cell membranes

of the SCN neurons (98). However, other studies reported that estradiol treatment of SCN explants from PER2:LUCIFERASE mice has no effect on the period and amplitude of the circadian oscillations (9, 99). Alternatively, estradiol may alter rhythms in running activity via indirect effects on the medial POA or striatum (100, 101).

Pituitary

Pituitary gonadotrophs express ER α , and estradiol has been proposed to exert a direct negative feedback effect on gonadotropin secretion (102–104). Chronic treatment with estradiol induces negative feedback effects on gonadotrophin secretion after GnRH supraphysiologic stimulation in ewes (105) or humans (106). Interestingly, a recent study reported that mice with a selective deletion of ER α in pituitary gonadotroph cells had elevated serum LH and estradiol values, and displayed irregular estrous cycles punctuated by prolonged periods of disorganized cycling, pointing to ER α participation in the estradiol negative feedback at the pituitary level (107). It is worth mentioning that this phenotype was much less severe than the one observed after a total ER α deletion. Although the role of Kiss/Kiss1R at the pituitary is still the subject of debate, it is interesting to note that the activation of pituitary ER α up-regulates *Kiss1* expression, whereas chronic exposure to estradiol down regulates *Kiss1R* expression on pituitary gonadotrophs (108).

Uterus

The uterus expresses high levels of ER α , and is an important site for the estrogenic control of reproductive physiology (109, 110). Estradiol, together with progesterone, regulates uterine growth and differentiation, which in turn control embryo-endometrial interactions during early pregnancy (110). Furthermore, estradiol treatment also shortens the period of the circadian clock in the uterus (99).

Daily and Circadian Regulations

Role of the Suprachiasmatic Nuclei

The Hypothalamic Suprachiasmatic Nuclei Host the Master Biological Clock

Most biological functions, including female reproduction, are synchronized to the daily variation of environmental factors. Among these factors, the recurring light/dark cycle is the most predictable environmental cue used by mammals to adjust their behavior and physiology appropriately. The mechanisms by which light and dark synchronize biological functions involve the master biological clock located in the hypothalamic SCN and a retino-hypothalamic tract, which forwards the non-visual light to the SCN.

The demonstration that a biological clock located in the basal hypothalamus was driving circadian rhythms came from experiments showing that SCN lesions in rodents abolished circadian rhythms in locomotor activity, which were restored following exogenous SCN implants (111, 112). The circadian activity of SCN neurons relies on a complex molecular system cycling endogenously with a period of about 1 day (*circa dies*). This molecular clockwork is composed of transcription–translation

loops, which are now well described (113, 114). Dimers of the CLOCK and BMAL1 proteins bind to a specific E-box promoter to induce the transcription of four clock genes *Per1*, *Per2*, *Cry1*, and *Cry2*, which after translation produce proteins which form dimers to repress their own transcription by competing with the CLOCK/BMAL1 binding. Following degradation of the inhibitory proteins, the transcription–translation loop starts over for another circadian cycle. When SCN explants or dissociated cells are kept *in vitro*, the endogenous circadian oscillations continue for weeks or months, providing that the culture medium gets enough nutrients and oxygen for the cell metabolism. A very interesting animal model used to demonstrate these sustained endogenous oscillations is the PER2:LUCIFERASE mice, where the expression of the *luciferase* gene is driven by the *Per2* promoter (115, 116). When explants or dissociated cells of PER2:LUCIFERASE mouse SCN are placed in a culture medium containing luciferine, the rhythmic expression of PER2 drives a rhythmic expression of luciferase, which by oxidizing luciferin causes the emission of a bioluminescent signal with a circadian period.

In order to achieve its role in adjusting biological functions with the astronomical daily cycles, the SCN circadian activity has to be synchronized with the time of day and transmit this timing information to the rest of the body. Light has long been known to be the main synchronizer of the SCN circadian clock, but interestingly it uses a specific non-visual pathway, which includes melanopsin-containing intrinsically photosensitive retinal ganglion cells projecting directly to the SCN (117, 118). Upon light activation, these ganglion cell terminals release glutamate and pituitary activating cAMP peptide, which change the phase (synchronizes) of the circadian clock. The synchronizing property of light depends on the time of application during the day, and the characteristics of the phase responses depend on species. The astronomical light/dark alternation synchronizes the circadian clock in order to attain a daily rhythm of a precise 24-h period. The CLOCK/BMAL1 dimers not only activate canonical clock gene expression, but other clock-controlled genes whose promoters display E-boxes and therefore undergo rhythmic expression. This mechanism was first demonstrated for the gene encoding AVP, an important output of the SCN clock (119). Levels of SCN AVP mRNA are markedly higher during the day than at night, but in *Clock* KO mice the SCN AVP rhythm is strongly dampened (120).

SCN Lesion or Clock Gene Mutations Alter the Reproductive Cycle

Various experiments aiming at impairing clock function were performed to delineate whether functional SCN neurons are required for the daily timing of the LH surge, mostly in female rodents. Early experiments of SCN lesions (121) or SCN-POA neuronal connection cut (122) resulted in an impaired LH surge and estrous cyclicity in female rats. Furthermore, female mice carrying mutations of *Clock* or *Bmal1* displayed disrupted estrous cycles (123–127). *Clock*^{−/−} mutant mice, for example, have extended and disrupted estrous cycles under both a light/dark cycle and during continuous darkness. In humans, it was reported that women with single-nucleotide polymorphisms in the ARNTL (*Bmal1*) have more miscarriages and less

pregnancies than those without (128). Although these experiments have pointed to a crucial role of the SCN in the proper timing of estrous cyclicity, reproductive impairment following clock gene mutations could as well result from peripheral clock desynchronization (see Other Clocks in the Reproductive System). Interestingly, the reproductive phenotypes of young Clock⁻, Bmal1⁻, or Per1/Per2-mutated mice resemble that of middle-aged (over 10-month old) wild-type mice, with increased length and decreased frequency of estrous cycles (129, 130). These observations indicate that alterations in central or peripheral clocks may lead to advanced reproductive senescence (130).

The preovulatory LH surge is initiated by a SCN-derived stimulatory signal, at a time closely preceding general activity onset. However, this signal is effective at stimulating GnRH neurons to produce the LH surge only when estradiol concentrations have reached a critical threshold. Prior to the day of proestrus, the developing ovarian follicles secrete insufficient estradiol to fulfill this criteria and therefore, the SCN signal does not trigger the LH surge (56). The occurrence of the daily stimulatory SCN signal can be unmasked by implanting female rodents with estradiol capsules that result in proestrus concentrations of this hormone; in this case, a LH surge occurs every day (3, 56, 131).

SCN Neuropeptides Involved in the Timing of the GnRH/LH Surge

Transplant of fetal SCN tissue into bilaterally SCN-lesioned hamsters restores locomotor, but not endocrine rhythms in the absence of neural outgrowth, suggesting that intact neural connections are required for endocrine rhythmicity, whereas behavioral rhythms can be supported by a diffusible signal (132). Neuroanatomical studies have pointed to two putative SCN neural outputs signaling daily information to the reproductive axis, AVP and vasoactive intestinal peptide (VIP). Early experiments have identified SCN-originating, VIP-containing fibers contacting GnRH neurons (133, 134), which express the VIP receptor VPAC2 (135). However, more recent studies indicate that the SCN signals the time of day to GnRH neurons indirectly via AVP fibers projecting to the Kp neurons of the AVPV (78, 89).

Anterograde tracing studies show that a number of AVP-containing axons originating in the SCN make appositions to Kp neurons, whereas very few or no VIP terminals were found apposed to Kp neurons (87, 89). Furthermore, AVP is released with a peak coinciding with the onset of the LH surge (136) and AVPV Kp neurons express V1a receptors (87). Interestingly, the AVP input to Kp neurons is sensitive to estradiol since estradiol treatment significantly increases the number of AVP terminal appositions on individual Kp neurons (89) and circadian expression of V1a mRNA is abolished in ovariectomized animals (88). Furthermore, AVP signaling onto Kp neurons is critically dependent on circulating estradiol as AVP no longer activates Kp neurons in ovariectomized mice, an effect that is fully restored by estradiol treatment (90). Altogether, these results are consistent with the hypothesis that Kp neurons located in the rodent AVPV receive daily information from the SCN via an AVPergic monosynaptic pathway, a signal which is modulated (gated) by circulating estradiol (Figure 1A).

In line with these neuroanatomical observations, earlier physiological experiments pointed to a functional role of AVP in the GnRH/LH surge timing, even though the importance of Kp neurons was not yet known. Inhibition of AVP signaling with a V1a antagonist resulted in a reduction in the estradiol-induced LH surge (137), while intracerebroventricular infusion of AVP in SCN-lesioned, ovariectomized, and estradiol-treated rats was able to induce an LH surge (138). Furthermore, in co-cultures of POA and SCN, the GnRH surge was coordinated with the rhythm in AVP, but not VIP, and administration of AVP, not VIP, to preoptic explants in the presence of estradiol significantly increased GnRH release, providing further evidence for an important role of AVP in the LH surge generation (139). Finally, a recent study reported that intracerebroventricular administration of AVP in female Syrian hamsters activates Kp neurons similarly in the early or late part of the day, while in the same animals GnRH neurons are activated only late in the day (87). This observation was further confirmed by *in vitro* electrophysiological recordings of Kp-GFP neurons showing that AVP increases the firing rate of most Kp neurons during proestrus, independently of the time of day (90). Altogether these findings indicate that AVP activates Kp neurons every day, and the daytime gating of the GnRH/LH surge does not take place through SCN AVP–AVPV Kp signaling but rather downstream at the AVPV Kp–POA GnRH signaling.

A significant role of the SCN-derived VIP output in female reproduction should not be excluded (Figure 1A). Indeed, VIP afferents on GnRH neurons are sexually dimorphic, with female rats exhibiting higher VIPergic innervation than males (134). Furthermore, central administration of VIP antiserum reduces the LH surge (140), while central infusion of VIP is able to rescue the LH surge in middle-aged female rats (141). Finally, blocking the VPAC₂ receptor attenuates GnRH neuronal cell firing during the afternoon surge in female, estradiol-treated mice (142). A recent study reported that the SCN-derived VIP neurons project to RFRP-3 neurons and central administration of VIP markedly suppresses RFRP-3 cellular activity in the evening, but not the morning, therefore indicating a specific role of VIP on neurons expressing RFRP-3, a neuropeptide thought to participate in the circadian-timed removal of estradiol negative feedback (143).

Other Clocks in the Reproductive System

It has long been thought that the SCN-driven outputs are the sole source forwarding circadian signals to the female reproductive system. However, a growing body of evidence now suggests that structures and organs that are part of the gonadotropic axis might also play an intrinsic role in the timing of female reproduction.

In mammals, the timing system is now described as a multioscillator hierarchy of coordinated and synchronized cell and tissue clocks (144). The use of *Per1-luc* transgenic rats (145) and *Per2:LUCIFERASE* transgenic mice (115), where the *Per1* or *Per2* promoter drives expression of the *luciferase* gene, was decisive for the demonstration that non-SCN central structures and peripheral organs can sustain endogenous circadian oscillations. Thus, central structures (e.g., olfactory bulb, ARN, and retrochiasmatic area) and peripheral organs (e.g., liver, lung, heart, and kidney) are able to display endogenous-sustained circadian rhythmicity. The phase and the period of these “peripheral” clocks are tissue

characteristic and different from those of the “central” clock of the SCN. The strength of their endogenous oscillations is often lesser than that of the SCN since according to tissues (and transgenic mice) the oscillations last from 2 to 20 cycles on average, whereas those of SCN can last several months. The oscillations generated by the peripheral clocks are independent of the SCN activity (they persist in SCN-lesioned animals) but their rhythms appear synchronized by the master clock, which is sometimes referred to as the conductor of the organism’s multi-oscillatory network. In the context of such a complex circadian network, recent evidence now suggests that the hypothalamo-pituitary gonadal axis is also a functional multi-oscillatory axis. Indeed, reproductive tissues from hypothalamic Kp and GnRH neurons down to the ovaries and the uterus display endogenous circadian oscillations of clock genes, as explained below. However, the functional role of these reproductive clocks with regards to the timing of reproduction (ovulation, implantation, and parturition) has yet to be determined.

Kisspeptin Neurons

Daily and circadian activities of Kp neurons in the AVPV area have been investigated in female rodents because of their strategic position between the integration of SCN-derived AVP input on one hand (87, 89) and the triggering of the preovulatory GnRH/LH surge on the other (19). Additionally, the modulatory effect of circulating estradiol on the daily activity of Kp neurons has also been investigated because of the potent effect of estradiol on Kp synthesis (77). Under high circulating estradiol levels, either in proestrus or in ovariectomized + estradiol-supplemented rodents, Kp neuronal activity (as seen by c-FOS activation) and *Kiss1* mRNA are significantly increased about 3 h before lights off, thus 2 h before the LH surge (9, 86–88). Furthermore, we recently reported that Kp immunoreactivity is markedly but transiently decreased at the same time (9). In contrast, in low circulating estradiol conditions, in diestrus or ovariectomized animals, the daily variation in neuronal activity, *Kiss1* mRNA and Kp immunoreactivity is abolished or strongly dampened (9, 86, 88). The daily activation of Kp neurons is triggered by the SCN AVP input since central injection of AVP induces c-FOS in Kp neurons and increases *Kiss1* mRNA (87). Although AVP is released from the SCN neurons in the afternoon (146), AVP can activate Kp neurons in the morning or in the afternoon, indicating that the daily control of the LH surge is not gated by the AVPV Kp neurons (87). A recent study recording Kp neuron electrical activity confirmed that Kp neuron responsiveness to AVP depends on the concentrations of estradiol (90). Altogether these data indicate that under high circulating estradiol (when oocytes are mature enough to be released), Kp neurons can be activated by the SCN-derived AVP to increase Kp synthesis and release in order to induce GnRH neuronal activation and the downstream LH surge. In addition to the AVP activation of Kp neurons, we recently reported that these neurons host an intrinsic circadian clock, named Kiss-Clock (9). A preliminary study reported that the clock genes *Per1* and *Bmal1* are expressed in the rat AVPV, but their cellular localization was not established (88). We further demonstrated that virtually all AVPV Kp neurons express the PER1 protein (Figure 1B) with a daily rhythm both in proestrus

and diestrus, but with a phase delay of about 3 h in diestrus as compared to proestrus (9). Furthermore, we reported that isolated Kp-expressing AVPV explants from PER2:LUCIFERASE mice display endogenous circadian oscillations with a period of 23.2 h (thus 1 h shorter than the SCN circadian period of the same mice), confirming the presence of an intrinsic circadian oscillator in AVPV Kp neurons (9) (Figure 1C). Remarkably, the period of this circadian clock is increased by 1 h in the presence of estradiol in the culture medium (Figure 1C), which is in line with the observed phase difference in PER1 expression according to the estrous stage. In contrast, the period of the SCN clock is not altered by environmental estradiol either *in vivo* or *in vitro* (9). The role of this Kiss-Clock has yet to be established. However, according to previous studies one might hypothesize that it could time sensitivity to estradiol since *ERα* gene expression can be regulated by the BMAL1/CLOCK dimer (147), or it could also impact *Kiss1* gene expression since a circadian transcriptional factor, albumin D-site binding protein (Dbp), was reported to trigger *Kiss1* transcription via the D-box (148).

GnRH Neurons

Because of their pulsatile activity and critical role in timing the LH surge, GnRH neurons were the first in the reproductive system to be reported to express clock genes and display circadian activity. Indeed, all core clock genes (*Clock*, *Bmal1*, *Per1/2*, and *Cry1/2*) are expressed and cycle with a circadian period in both GT1-7 GnRH neuronal cell lines and GnRH neurons (149–152). Disruption of the circadian clock by transient expression of *Clock* Δ 19 in GT1-7 cells decreases the GnRH pulse frequency, while overexpression of *Cry1* in the same cells increases GnRH pulse amplitude (149). The GnRH clock could also regulate the timing of the neuronal sensitivity to upstream inputs. Indeed, the ability of VIP to activate GnRH neurons depends on the time of day and the estradiol environment (153), and the sensitivity of GT1-7 cells to release GnRH upon Kp or VIP treatment is time dependent (151). This time-dependent sensitivity may explain why central infusion of Kp fails to advance the onset of the LH surge in either naturally cycling or ovariectomized estradiol-supplemented female rodents (154, 155). Finally, the stimulatory and synchronizing effects of Kp on GnRH release are reduced in preoptic explants of *Bmal1* KO mice (35). Therefore, the circadian clock in GnRH neurons may provide a time-keeping mechanism to appropriately release GnRH under Kp, and possibly VIP, stimulation.

Pituitary Gland and Gonadotroph Cells

The pituitary, as a whole, was among the first peripheral oscillators found to display strong sustained circadian oscillations with a circadian period of about 23.8 h in *Per1*- or PER2-luciferase transgenic rodents (115, 145). It was further established that all clock genes expressed a daily rhythm in the whole pituitary but with a different profile according to the estrus stage (127, 156). The pituitary gland is made of different cell types, which could host several circadian oscillators with different phases. Expression of all cognate clock genes was identified in the alphaT3-1 gonadotroph cell line (147) and GnRH activation was reported to selectively increase *mPer1* expression in gonadotroph cells (157).

Furthermore, the gene coding for the GnRH receptor contains non-canonical E-box promoter elements and *Bmal1* knockdown in a gonadotrope cell line reduces GnRH receptor mRNA (147). These studies raised the hypothesis that an intrinsic clock in gonadotrophs could directly regulate GnRH signaling and LH surge timing. To test this hypothesis, a specific BMAL1 KO disruption was performed in the gonadotrophs (127). The mutated mice still displayed a preovulatory LH surge and estrous cyclicity (although with a significant increase in cycle length variance) and the average time of puberty and fertility performance was not altered. Taken together, these data suggest that the intrinsic clock in gonadotroph cells is dispensable for LH surge regulation but contributes to estrous cycle robustness (127).

Ovaries

The ovarian circadian clock is very well documented in many mammalian and non-mammalian species and its function has been thoroughly investigated both *in vivo* and *in vitro* (158–161). Each cell type of the ovary, including theca cells, granulosa cells, and oocytes have a circadian clock (162). Further analyses reported that clock gene rhythms are only observed in mature granulosa and luteal cells, indicating that these rhythms are activated at a specific stage of follicle development, possibly under the control of FSH acting as a synchronizer of follicular cell activities (163). Ovarian physiology is strongly regulated by gonadotropins, and current studies indicate that LH stimulates various clock genes in the ovaries (159, 164). Furthermore, the endogenous rhythm of Per-driven oscillations in isolated ovaries is significantly shifted by LH and FSH indicating that the ovarian circadian clock is entrained by hormonal signals from the pituitary (160). Recently, a study reported that mice with a conditional KO of *Bmal1* in steroidogenic cells show severe deficits in implantation success and compromised progesterone secretion (165). A previous study demonstrated a circadian rhythm in ovarian sensitivity to LH with a greater ovarian response at night as compared to day, indicating that the ovarian circadian clock may set its responsiveness to the LH surge (161). Finally, various ovarian genes, including those encoding for the LH receptor and enzymes involved in steroid hormone biosynthesis, display circadian rhythms in granulosa cells and these rhythms are altered following the silencing of *Bmal1* expression (163). Altogether, these findings indicate that the clock in the ovary may be involved in the timing of ovulation, steroid hormone synthesis, and follicular growth and differentiation.

Uterus and Oviduct

Global knockout of the *Bmal1* or *Clock* gene disrupts implantation, increases fetal reabsorption during pregnancy, and leads to a high rate of full-term pregnancy failures (124, 166). Early in 2002, clock genes were found to be expressed in the uterus and oviduct of mice (167). Furthermore, the oviduct was reported to display a daily rhythm of several clock genes and clock-controlled gene (168). In the uterus, the presence of sustained endogenous clock oscillations was demonstrated in tissue explants of PER2:LUCIFERASE mice (99). These uterine oscillations were sustained even during pregnancy suggesting that embryos may be submitted to the maternal

clock *in utero* (169). Interestingly, the period of the uterine clock oscillations changes according to the estrous stage and it is decreased when the tissue is incubated with estradiol (99). Additionally, a targeted deletion of *Bmal1* in the myometrium indicates a role for myometrial *Bmal1* in maintaining normal timing of parturition (170). Although additional studies are required to determine the physiological role of the uterine and oviduct clocks, the data obtained so far suggest that the developing embryo may be subjected to rhythmic changes in the oviduct during transit to the uterus and in the uterus during pregnancy.

Shift-Work Consequences on Reproductive Cycles and Fertility

Shift Work

The modern 24-h-functioning society requires that an increasing number of employees work outside of the natural active period, in shifted conditions. According to the International Labor Organization (ILO; 1990), working in shifts is “a method of organization of working time in which workers succeed one another at the workplace so that the establishment can operate longer than the hours of work of individual workers” at different daily and night hours. Under a fixed-shift system, working time can be organized in two or three shifts: the early, late, and/or nightshifts. Under a rotating-shift system, workers might be assigned to work shifts that vary regularly over time.

Over the last 20 years in United States, almost 27% of men and 16% of women experienced shift work (171). In 2012, 15% of French workers, including 9.3% women, were working under shift work either occasionally (8%) or permanently (7.4%). An increasing number of analyses report that alteration in working schedule is often associated with an increased risk of developing cardiovascular/metabolic/gastrointestinal disorders, some types of cancer, and mental disorders including depression and anxiety (172–174). Hence, in 2007, shift work was reclassified from a possible to a probable human carcinogen (class 2A) by the International Agency for Research on Cancer. A French law passed on December 20, 2014 listed shift work as a risk factor increasing professional arduousness.

Given the importance of the circadian systems in the regulation of female reproduction, and given the fetal exposure to the maternal daily rhythms in temperature, substrates, and hormones, female shift workers may display reproductive alterations, such as an increased risk of irregular menstrual cycles, endometriosis, miscarriage, low birth weight, or pre-term delivery (175–177). Such disturbances may result from altered SCN clock synchronization with rest-activity and feed-fast cycles and/or internal desynchronization amongst peripheral clocks, especially those of the reproductive axis. Indeed, a recent study reported that peripheral clock genes in lymphocytes of shift workers are strongly altered as compared to day workers (178). Additionally, recent animal studies have shown that the functioning of fetal clocks depends on maternal hormones (179) and possibly feeding and activity, then maternal circadian disruption during pregnancy may lead to fetal SCN and peripheral clocks desynchronization.

In addition to the effect of circadian dysregulation, it should be kept in mind that shift work-related alterations in other daily functions, particularly food intake and sleep, may indirectly impact female reproduction. Thus, obesity, which is often associated with shift work, has a strong impact on reproductive performance (180) and sleep disturbance in prepubertal girls can alter estradiol-dependent pubertal development (181).

Modeling Shift Work in Rodents

Shift work is a very complex situation and therefore, it is difficult to design animal model conditions that mimic human shift work. A recent review listed four relevant models that use altered timing of food intake, activity, sleep or light exposure, or a combination of several (182).

Very few *in vivo* animal studies have investigated the alteration of fertility or the LH surge after a shift in the light/dark cycle or a photoperiod change (6, 56, 183). In one study, female Syrian hamsters were submitted to a 3-h phase advance or delay (183). When the phase advance was applied between 1 and 3 days before estrous, the LH surge was not fully resynchronized to the new schedule, even after 3 days. However, when hamsters were submitted to a 3-h phase delay, the LH surge was synchronized to the dark onset more rapidly. Similarly, in ovariectomized transgenic GnRH-GFP mice, a dark phase advance led to an advance in the LH surge (56). When the photoperiod length was modified in female hamsters, the timing of the LH surge was shifted in a similar manner to the nocturnal onset of locomotor activity (6).

In mice, exposure to either phase advances or delays at the beginning and throughout pregnancy leads to a significant decrease in pregnancy success (184). Interestingly, an *in vitro* study analyzed the effect of a 6-h phase advance on endogenous circadian oscillation of the SCN and various peripheral clocks and found that the ovarian clock was not fully resynchronized 6 days after the phase shift (185).

Although these few studies indicate that shifts in light/dark cycle alter the timing of the preovulatory LH surge and the synchrony amongst reproductive clocks, it is obvious that new animal studies have to be developed in order to understand the mechanisms underlying the various effect of shift work on reproduction and fertility in females.

Reproductive Consequence in Women Under Shift Work

A number of studies have investigated the relationship between fertility and shift work or night work in women working in pharmaceutical industries, hospitals, slaughter houses, and canneries (175, 178, 186–200). Although human studies are limited in their use for understanding causality and underlying mechanisms of health consequences of shift work, most of the above studies have reported a negative impact of shift work on fertility. However, there is a large heterogeneity among these analyses especially regarding the fertility criteria examined: body temperature curve, menstrual disorders, time to get pregnant, etc. Furthermore, it is important to stress that clinical or biological criteria can be misinterpreted since irregular cycles, as an indicator of the reproductive axis sensibility to shift work, may have no correlation with subfertility and pregnancy capacity (197).

Using body temperature curves to follow menstrual cycles, shift work was found to be associated with higher rates of short cycles and inadequate luteal phases (188). Furthermore, a higher prevalence of menstrual disorders is often found in female shift workers as compared to the non-shift workers. For example, in the most relevant studies including the largest populations, irregular cycles are reported in 12–20% of shift workers and 7–10% in non-shift workers (175, 192). In a large Danish population (17,531 daytime workers and 3,907 shift workers), it was reported that fixed evening and fixed night female workers took longer to get pregnant with adjusted odds ratio around 0.80, compared to daytime workers, but there was no unequivocal evidence of a causal association between shift work and subfecundity since this reduction may be mediated by pregnancy planning bias or differential options for sexual contacts (187). Only a few studies have examined reproductive hormones with various conclusions. It has been reported that FSH and LH levels are not different between shift and day workers (190, 192), but a single measurement of LH without a gynecologic examination and cycle characterization is difficult to interpret. The levels of 17- β -estradiol were found to be significantly increased (178, 201) in female shift workers possibly due to a prolonged follicular phase (186). Some studies have reported no significant relationship between shift work and subfertility or dysmenorrhea (196–200). However, among these studies, one has only a few women included with a surprising 35% of control women displaying irregular cycles (196). Another study reported no significant subfertility in shift-working women 1 year after birth control termination but yet, these women displayed a delay to get pregnant twice as long compared to the day-working group (199).

The pineal hormone melatonin, whose nocturnal production is profoundly affected by shifts in light/dark conditions, has been proposed to display potential anti-estrogenic effects (202, 203). Urinary melatonin excretion tends to be lower with a delayed peak of production during shift work (190, 193, 201, 204). The combination of inhibition on melatonin secretion with estradiol mistiming has been proposed to be involved in the hormone-related cancers observed in night shift workers (204, 205).

The conflicting results regarding the negative effect of shift work on female reproduction probably reflect large differences in the shift-work schedules, duration, and age of exposure, with a high number of confounding factors (like stress, fatigue, obesity, etc.) as well as methodological limitations (206, 207). Despite these considerations, cycling disorders should be considered as a sensitivity or intolerance to shift work. Shift work during pregnancy has adverse effects including increased risk of miscarriage (208), although this is somewhat controversial (209). Yet, most authors recommend avoiding shift work during pregnancy.

Conclusion

Daily and estrogenic regulations of female reproduction allow the timing of ovulation to coincide with optimal reproductive tract functioning (oocyte maturation and receptive reproductive tract), maximal arousal (general activity and sexual motivation), and the best environmental conditions (food resources and stress level). These general coordinations confer maximum adaptive

advantage to insure the success of this high energy-demanding reproductive function. Although the central role of the master SCN clock in the daily regulation of the LH surge has been well documented, the recent evidence that other peripheral clocks are located all along the gonadotropic axis, from Kp and GnRH neurons to the ovaries and uterus, raises the question of their role in the timing of reproduction. The latest findings indicate that these local clocks may optimize circadian cell sensitivity to upstream signals and set appropriate timing of the downstream reproductive responses. Notably, application of phase shifts leads to different rates of clock resynchronization between the SCN and reproductive organs, suggesting internal desynchronization of the reproductive axis, as seen in other functional axes. In our current society, where a significant number of female workers are working night or evening shifts, the delicate timing of organization in the reproductive network can easily be disrupted. While numerous studies have reported negative consequences of shift work on metabolic and cardiovascular functions as well as cancer

occurrence, there are surprisingly few epidemiologic studies in humans and mechanistic studies in animal models reporting the incidence of shift work on female fertility. Future studies in the field should, therefore, investigate the impact of daily rhythm alterations, as observed under shift-work conditions, jet lag, or sleep disturbance, on reproductive cycle and fertility both in animal models and humans.

Author Contributions

VS and TB contributed equally to the writing of this review.

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Neuropeptide co-expression in hypothalamic kisspeptin neurons of laboratory animals and the human

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Hypothalamic peptidergic neurons using kisspeptin (KP) and its co-transmitters for communication are critically involved in the regulation of mammalian reproduction and puberty. This article provides an overview of neuropeptides present in KP neurons, with a focus on the human species. Immunohistochemical studies reveal that large subsets of human KP neurons synthesize neurokinin B, as also shown in laboratory animals. In contrast, dynorphin described in KP neurons of rodents and sheep is found rarely in KP cells of human males and postmenopausal females. Similarly, galanin is detectable in mouse, but not human, KP cells, whereas substance P, cocaine- and amphetamine-regulated transcript and proenkephalin-derived opioids are expressed in varying subsets of KP neurons in humans, but not reported in ARC of other species. Human KP neurons do not contain neurotensin, cholecystokinin, proopiomelanocortin-derivatives, agouti-related protein, neuropeptide Y, somatostatin or tyrosine hydroxylase (dopamine). These data identify the possible co-transmitters of human KP cells. Neurochemical properties distinct from those of laboratory species indicate that humans use considerably different neurotransmitter mechanisms to regulate fertility.

Keywords: CART, dynorphin, hypothalamus, kisspeptin, neurokinin B, reproduction, substance P

REPRODUCTIVE SIGNIFICANCE OF KP NEURONS IN MAMMALS

Hypothalamic neurons synthesizing kisspeptin (KP) play a pivotal role in the central regulation of puberty and reproduction. Inactivating mutations of the genes encoding for KP (*KISS1*) (Topaloglu et al., 2012) or its G-protein-coupled receptor (*KISS1R*; previously called GPR-54) (De Roux et al., 2003; Seminara et al., 2003) cause hypogonadotropic hypogonadism in humans. Impaired fertility has also been observed in *Kiss1* (D'anglemont De Tassigny et al., 2007; Lapatto et al., 2007)- and *Kiss1r* knock-out mice (Funes et al., 2003; Seminara et al., 2003) suggesting the highly conserved reproductive significance of KP/*KISS1R*-signaling in mammals. KP is a potent stimulator of adenohipophysial LH and FSH secretion (Navarro et al., 2005a,b). This action involves gonadotropin-releasing hormone (GnRH) and can be prevented with the GnRH receptor antagonist acyline (Gottsch et al., 2004; Shahab et al., 2005). The major effect of KP on GnRH-synthesizing neurons is direct. In various species, (i) KP-immunoreactive (IR) fibers establish appositions to GnRH neurons (Kinoshita et al., 2005; Clarkson and Herbison, 2006; Ramaswamy et al., 2008; Smith et al., 2008), (ii) GnRH cells express *Kiss1r* (Irwig et al., 2004; Han et al., 2005; Messenger et al., 2005), (iii) GnRH neurons responds to KP with cFos expression (Irwig et al., 2004; Matsui et al., 2004) and depolarization (Han

et al., 2005; Dumalska et al., 2008; Pielecka-Fortuna et al., 2008), (iv) the GnRH-specific *Kiss1r*^{-/-} mice are infertile (Novaira et al., 2014) and (v) the infertile phenotype of global *Kiss1r*^{-/-} mutant mice can be rescued via the selective reinsertion of *Kiss1r* into GnRH neurons (Kirilov et al., 2013).

FUNCTIONAL SUBSETS OF KP NEURONS IN LABORATORY SPECIES AND THE HUMAN

In a variety of mammals, two major populations of KP-synthesizing neurons exist in the anterior preoptic area and the arcuate nucleus (ARC), respectively (Lehman et al., 2010a). In rodents, the anterior preoptic cell group occurs as a periventricular continuum within the anterior periventricular (AVPV) and the periventricular preoptic (PVpo) nuclei (Clarkson and Herbison, 2006), together referred to as the KP neuron population of the rostral periventricular area of the third ventricle (RP3V) (Clarkson et al., 2008). Both KP cell populations are also detectable in the human, with the bulk of neurons in the infundibular (=arcuate) nucleus (Inf) (Romero et al., 2007; Hrabovszky, 2013). KP neurons in the rodent RP3V and both KP cell groups in the human are sexually dimorphic, with higher cell number in females than in males (Clarkson and Herbison, 2006; Kauffman et al., 2007; Hrabovszky et al., 2010, 2011). Information accumulated in recent years indicate that both cell populations

contain other neuropeptides and classic transmitters, in addition to KP. This review article discusses the available literature about these co-transmitters of KP neurons in laboratory species and humans, as also summarized in **Figure 2**.

CO-TRANSMITTERS AND THEIR RECEPTORS IN KP NEURONS OF LABORATORY SPECIES

KP NEURONS OF THE ANTERIOR PREOPTIC REGION

KP cells in the RP3V of female rodents have been implicated in positive estrogen feedback to GnRH neurons (Adachi et al., 2007; Herbison, 2008; Robertson et al., 2009). Immunohistochemical (IHC) and *in situ* hybridization (ISH) studies revealed that KP neurons in the RP3V express other neuropeptides as well as classic neurotransmitters.

Met-enkephalin (mENK)

Neurons IR to the proenkephalin (pENK)-derived opioid mENK overlap with KP-IR cells of the RP3V. Porteous et al. reported that, in adult female mice, dual-phenotype KP/mENK cells represent 28–38% of all KP-IR and 58–68% of all mENK-IR neurons in the AVPV and PVpo, respectively. These neurons give rise to dual-labeled axon varicosities which project to the preoptic area, the anterior hypothalamus and the ARC (Porteous et al., 2011).

Galanin

Recent ISH and IHC studies have established that galanin is also present in a subset of KP neurons in the RP3V of the mouse (Porteous et al., 2011; Kallo et al., 2012). In estrogen-treated ovariectomized mice, galanin was detected in 87%, and galanin mRNA in 38%, of KP neurons (Kallo et al., 2012). A lower incidence of colocalization was reported by Porteous et al.; in their study dual-labeled cells represented 7% of all KP-IR and 21% of all galanin-IR neurons both in the AVPV and PVpo of colchicine-pretreated female mice (Porteous et al., 2011).

Tyrosine hydroxylase (dopamine)

Tyrosine hydroxylase (TH) is a key and rate-limiting enzyme in catecholamine synthesis. Similarly to KP cells, TH-containing neurons in the RP3V are sexually differentiated in rodents (Kauffman et al., 2007; Clarkson and Herbison, 2011). In female rats, 20–50% of *Kiss1* mRNA-expressing cells in the RP3V, depending on the hormonal status, express *TH* mRNA (Kauffman et al., 2007). In female mice, 50% of KP cells contain TH immunoreactivity and vice versa, without noticeable variation between diestrus and proestrus. These KP/TH dual-phenotype neurons were also proposed to serve as the major source of dopamine in the synaptic regulation of GnRH neurons (Clarkson and Herbison, 2011).

GABA and glutamate

Dual-label ISH studies have established that about 20% of KP neurons in the mouse AVPV also express the mRNA encoding the glutamatergic marker type-2 vesicular glutamate transporter (vGluT2), whereas the mRNA of the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD-67) was expressed in 75% of KP neurons (Cravo et al., 2011). These data indicate that AVPV KP neurons use amino acidergic, in addition to peptidergic and dopaminergic co-transmission.

“KNDy” NEURONS IN THE ARC/Inf

Unlike the preoptic cell population, KP neurons of the ARC co-synthesize KP, neurokinin B (NKB) and dynorphin (Dyn) in several species (Burke et al., 2006; Foradori et al., 2006; Goodman et al., 2007; Navarro et al., 2011a; Bartzen-Sprauer et al., 2014), forming the basis for the “KNDy neuron” terminology (Lehman et al., 2010b). It is only becoming recognized lately that KNDy neurons do not consist of a homogenous cell population in that co-expression of the three KNDy peptides is only partial (Cheng et al., 2010; Hrabovszky et al., 2010, 2011, 2012; Overgaard et al., 2014). Morphological and electrophysiological studies provided evidence that KNDy neurons communicate extensively with each other (Burke et al., 2006; Foradori et al., 2006; Goodman et al., 2007; Navarro et al., 2011a,b; De Croft et al., 2013; Ruka et al., 2013). This local communication via NKB/neurokinin 3 receptor (NK3R) and Dyn/ κ -opioid receptor (KOR) signaling was proposed to play a critical role in the generation of episodic GnRH/LH pulses (Navarro et al., 2009; Ohkura et al., 2009; Wakabayashi et al., 2010).

In addition to playing a putative role in the regulation of pulsatile GnRH/LH secretion, KNDy neurons have been implicated in negative sex steroid feedback action. Accordingly, neurotoxic ablation of KNDy neurons in rats prevented the rise in serum LH after ovariectomy (Mittelman-Smith et al., 2012). In some species like the sheep and primates, KNDy neurons might also be involved in positive estrogen feedback regulation. KP neurons in the ARC of ovariectomized ewes respond with cFos expression to estradiol treatment (Smith et al., 2009) and female rhesus monkeys with a disconnected mediobasal hypothalamus continue to respond to estrogen with LH and FSH surges (Krey et al., 1975; Plant et al., 1979). In contrast with the data from sheep, KP neurons in the preoptic area, but not the ARC, are activated during the positive estradiol feedback in goats (Matsuda et al., 2014).

Moreover, as reviewed by Rance et al., KP neurons in the ARC/Inf are also involved in the control of thermoregulation and their dysfunction is likely to contribute to the generation of hot flashes during menopause (Rance et al., 2013).

KP neurons of the ARC co-contain other neuropeptides, neuropeptide receptors and classic neurotransmitters in different laboratory species, as discussed below.

NKB and its receptor NK3R

The high reproductive significance of NKB, product of the human *TAC3* and mouse *Tac2* genes, has been recognized recently (Lasaga and Debeljuk, 2011). Mutations in the *TAC3* or *TACR3* genes encoding for NKB and its receptor NK3R, respectively, lead to hypogonadotropic hypogonadism and infertility in humans (Guran et al., 2009; Topaloglu et al., 2009). In laboratory species, large percentages of ARC/Inf KP neurons contain NKB or NK3R, although the reported colocalization patterns vary largely by species, sex and age (Burke et al., 2006; Goodman et al., 2007; Navarro et al., 2009, 2011a; Amstalden et al., 2010; Cheng et al., 2010; Ramaswamy et al., 2010; Wakabayashi et al., 2010; Hrabovszky et al., 2011, 2012; Overgaard et al., 2014). For example, while 90% of KP-synthesizing neurons in ovariectomized mice expressed *Tac2* mRNA signal and virtually all contained *Tac3r* signal, estradiol replacement decreased the incidence of

NKB/Kiss1 co-labeled neurons to 53% and massively suppressed *Tac3r* mRNA expression (Navarro et al., 2009). In males, only half of the *Tac2*-expressing neurons expressed *Kiss1* mRNA, both in orchidectomized and testosterone-treated male mice (Navarro et al., 2011a). Similarly, a prominent group of NKB-only neurons was also detected in the caudal ARC in orchidectomized male, but not in ovariectomized female rats (Overgaard et al., 2014) and fibers single-labeled for NKB were also reported in the median eminence of female rats (True et al., 2011). The sex steroids estradiol (Navarro et al., 2009) and testosterone (Navarro et al., 2011a) regulate negatively *Tac2* and *Tac3r* expression in the ARC, which was proposed to decrease the activity of KNDy neurons by reducing a positive auto-feedback through NKB/NK3R signaling. Unlike in the above colocalization studies from rodents, only 40–60% of KP neurons co-expressed NKB immunoreactivity and NKB-only cells were not observed in the ARC of neonatally orchidectomized adult male monkeys (Ramaswamy et al., 2010).

While only NK1R and NK3R tachykinin receptors localized anatomically to KNDy neurons (Navarro et al., 2014), electrophysiological studies on male mice established that NKB stimulates the firing frequency of ARC KP neurons via activation of all three tachykinin receptor forms (NK1R, NK2R, NK3R) (De Croft et al., 2013). In contrast, in ovariectomized goats the NK3R receptor form plays the predominant role in the generation of GnRH pulses, with little, if any, contribution by NK1R and NK2R (Yamamura et al., 2015).

Dyn and its receptor KOR

The *Pdyn* gene product Dyn is the third KNDy peptide which was first colocalized with KP and NKB in the sheep; 95% of Dyn-IR cell bodies in ovariectomized and estrogen-treated ewes were also immunopositive for KP (Goodman et al., 2007). The extent of colocalization between KP and Dyn is also over 90% in the ARC of female mice, regardless of estrogen status (Navarro et al., 2009). ISH studies also revealed *KOR* mRNA, although only in relatively low subsets of KP neurons in female and male mice (Navarro et al., 2009).

Galanin

Galanin is co-expressed with KP not only in the RP3V (Porteous et al., 2011; Kallo et al., 2012) but also in the ARC (Kallo et al., 2012) of mice. In ovariectomized females, galanin mRNA was detected in 42.5%, and galanin immunoreactivity in 12.5%, of KP neurons (Kallo et al., 2012).

GABA and glutamate

ISH data indicate that KNDy neurons use amino acidergic co-transmitters and express *vGluT2* and *GAD-67* mRNAs (Cravo et al., 2011). Unlike KP cells of the RP3V which are mostly GABAergic (Cravo et al., 2011), the majority of KNDy neurons are glutamatergic (Cravo et al., 2011), in accordance with the IHC detection of *vGluT2* in NKB/Dyn neurons of male and female rats (Ciofi et al., 2006).

IMMUNOHISTOCHEMICAL PROFILING OF HUMAN KP NEURONS IN THE INF

While neurochemical data about the preoptic KP cell population of the human are currently unavailable, a series of recent

studies from our laboratory used immunofluorescent multiple-labeling to determine the phenotype of KP neurons in the Inf (Hrabovszky et al., 2010, 2011, 2012, 2013; Molnar et al., 2012; Skrapits et al., 2014). Colocalization experiments were carried out on autopsy samples from men of variable age groups as well as from postmenopausal women where expression is the highest for *KISS1* mRNA (Romero et al., 2007) and KP immunoreactivity (Hrabovszky et al., 2010). Procedures of tissue processing, technical measures to maximize signals and avoid false-positive colocalization results, and details of the confocal analysis were described in the original publications. Here we extended the colocalization experiments to several other neuropeptides in order to obtain a fingerprint of KP co-transmitters/modulators. Samples were used from both men and postmenopausal women, in view that age and sex have strong effects on neuropeptide levels of KP neurons (Hrabovszky et al., 2011; Molnar et al., 2012). For reference to studies and primary antibodies, see **Figure 2**.

NEUROPEPTIDES PRESENT IN HIGH PERCENTAGES OF HUMAN KP CELLS

Neurokinin B (NKB)

Similarly to the ARC of laboratory animals, the Inf encloses a large population of KP neurons that also synthesizes NKB (Hrabovszky et al., 2010). The density of KP-IR (and NKB-IR) neurons in the Inf as well as the extent of their co-expression were found to be highly sex-dependent (Hrabovszky et al., 2011) (**Figures 1A,B**) and age-dependent (Molnar et al., 2012). As reviewed recently (Hrabovszky, 2013), the overall incidences of KP-IR and NKB-IR cell bodies are highest in postmenopausal women, lower in aged men and the lowest in young men. The percentage of KP perikarya containing NKB is similar in postmenopausal women (71%), aged men (>50 years; 78%) and young men (<50 years; 73%), whereas the percentages of NKB-IR perikarya with KP are highest in postmenopausal women (84%; **Figure 1A**), somewhat lower in aged men (68%; **Figure 1B**) and quite low in young men (36%), indicating that KP expression in NKB neurons is highly sex- and age-dependent. While KP expression might be suppressed in NKB neurons by testosterone in young men, it starts to increase with the decline of this negative feedback in aged individuals. The highest KP level and colocalization percentage are found in postmenopausal women where the inhibitory effect of estradiol is absent. Of note, considerable subsets of the KP-IR and NKB-IR fibers in all models are single-labeled (Hrabovszky et al., 2011; Molnar et al., 2012; Skrapits et al., 2014) and only 8–10% of KP-IR and NKB-IR axons forming appositions to GnRH neurons in young men and 25–30% in postmenopausal women contained both KP and NKB (Hrabovszky et al., 2011; Molnar et al., 2012).

Substance P (SP)

The tachykinin peptide SP is derived from the *TAC1* gene and acts mainly via the NK1R. ISH studies by Rance et al. revealed that the *TAC1* and *TAC3* transcripts exhibit overlapping distribution in the Inf and both mRNAs increase remarkably after menopause (Rance and Young, 1991). Our laboratory used immunohistochemistry to analyze SP-IR neurons in the infundibular region (Hrabovszky et al., 2013). These studies demonstrated that the number and the staining intensity of SP-IR perikarya are

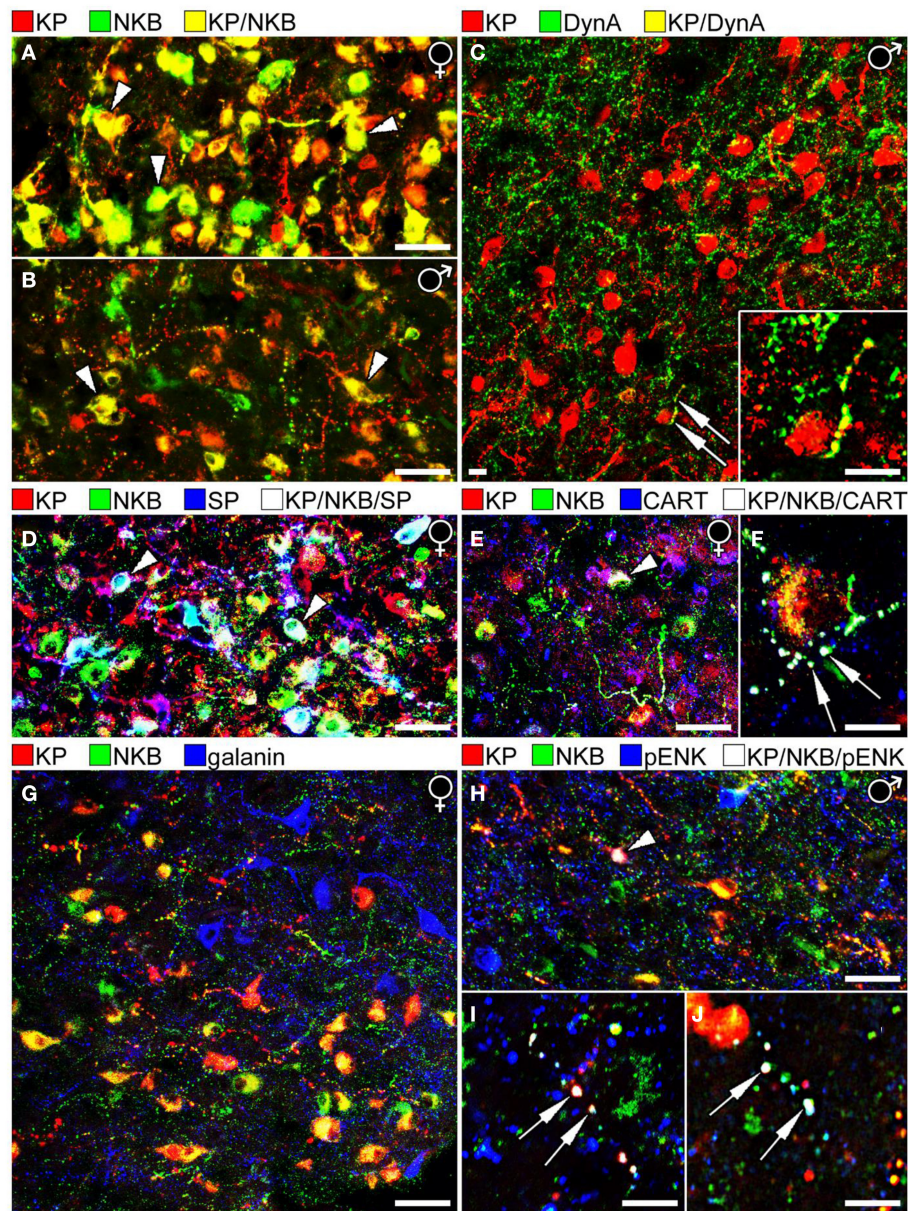


FIGURE 1 | Results of immunofluorescent studies to characterize the neuropeptide phenotype of human KP neurons in the Inf. (A,B) The highest numbers of KP and NKB neurons and colocalization percentages can be observed in the Inf of postmenopausal women **(A)**. Compare to the weaker labeling of the Inf from a 67 year-old men in **(B)**. Note that the Inf contains many single-labeled axons (red and green), in addition to dual-labeled ones (yellow) in both sexes. **(C)** Unlike KP neurons of laboratory animals, the majority of human KP neurons do not contain Dyn A immunoreactivity. A rare case of dual-labeled KP/Dyn A axon (arrows) is shown at higher power in the inset. **(D)** KP neurons often contain SP immunoreactivity in postmenopausal women. White cells labeled by arrowheads correspond to KP/NKB/SP triple-phenotype neurons (Hrabovszky et al., 2013). **(E,F)** Similarly, CART is

co-expressed in large subsets of KP and NKB neurons in postmenopausal women (Skrapits et al., 2014). Arrowhead in **(E)** and the two arrows in **(F)** point to a triple-labeled perikaryon and two axon varicosities, respectively. **(G)** The majority of neuropeptides tested in this study (see **Figure 2**), including galanin (blue), showed no colocalization with KP or NKB. **(H–J)** One exception was pENK which occurred in subsets of NKB-IR and KP-IR perikarya and fibers. Arrowhead in **(H)** points to a perikaryon, whereas arrows in **(I,J)** label axon varicosities that exhibit triple-neuropeptide phenotype (KP/NKB/pENK). The results shown in **(H,I)** vs. **(J)** were obtained with two different pENK antibodies (see text) to serve as positive control. (Note that the original color of the fluorochromes was changed so that KP is illustrated consistently in red). Scale bars: 40 μm in **(A,B,D,E,G,H)** and 12 μm in **(C,F,I,J)**.

significantly higher in postmenopausal women vs. age-matched men (Hrabovszky et al., 2013). We have shown a considerable overlap in the distribution of SP-IR, NKB-IR and KP-IR perikarya of postmenopausal women and we have established that SP

immunoreactivity is present in large subsets of the KP-IR and NKB-IR neurons (**Figure 1D**); 31% of KP-IR and 25% of NKB-IR perikarya in this study contained SP, whereas 16.5% of all labeled cell bodies exhibited triple-neuropeptide phenotype. Dual- and

| | Laboratory animal data | Human immunofluorescence data (Inf only) | Neuropeptide antibodies used in human studies |
|-----------------------------------|--|--|--|
| Co-transmitters in KP neurons | | | sheep anti-human KP-54 (GQ2) (Dhilllo et al., 2005) |
| | | | rabbit anti-human preproKP (AAS26420C; Antibody verify) |
| KNDy peptides | | | |
| Dyn | Reported in the ARC of sheep (Goodman et al., 2007), mouse (Navarro et al., 2009, 2011a), rat (Overgaard et al., 2014), hamster (Bartzen-Sprauer et al., 2014) and goat (Wakabayashi et al., 2010) | Extremely scarce colocalization in fibers in young men (Hrabovszky et al., 2012) | rabbit anti-porcine/human Dyn A (T-4268; Peninsula Laboratories) rabbit anti-Dyn B (IS-35) (Ciofi et al., 2006) |
| NKB | Reported in the ARC of sheep (Goodman et al., 2007), mouse (Navarro et al., 2009, 2011a), rat (Overgaard et al., 2014), hamster (Bartzen-Sprauer et al., 2014) and goat (Wakabayashi et al., 2010) and monkey (Ramaswamy et al., 2010) | Present in large subsets of KP neurons and fibers (Hrabovszky, 2013; Hrabovszky et al., 2010, 2011; Molnar et al., 2012) | rabbit anti-human proNKB (IS-681; IS-682) (Hrabovszky et al., 2010) mouse anti-human Tachykinin-3 (M-871-100; Biosensis Pty. Ltd) |
| Other neuropeptides | | | |
| AGRP | Not detected in the ARC of sheep (Goodman et al., 2007) | No colocalization in KP and NKB neurons (present study) | guinea pig anti-AGRP (GP-029-50; Biosensis Pty. Ltd) |
| α MSH, γ MSH (POMC) | Not detected in the ARC of mouse (Cravo et al., 2011) and sheep (Goodman et al., 2007) | No colocalization in KP and NKB neurons (present study) | sheep anti- α MSH gift from J. Tatro (Elias et al., 1998) |
| CART | Not reported | Colocalized in large subsets of KP and NKB neurons (Skrapits et al., 2014) | mouse anti-CART gift from J.S. Clausen (Vrang et al., 1999) rabbit anti-CART gift from M.J. Kuhar (Koylu et al., 1997) |
| CCK | Not detected in the RP3V of mouse (Porteous et al., 2011) | No colocalization in KP and NKB neurons (present study) | rabbit anti-CCK (IS-15/8) (Ciofi and Tramu, 1990) |
| GAL | Reported in both the RP3V and the ARC of mouse (Porteous et al., 2011; Kallo et al., 2012) | No colocalization in KP and NKB neurons (present study) | goat anti-galanin (EB09679; Everest Biotech Ltd) |
| mENK, pENK | Reported in the RP3V of mouse (Porteous et al., 2011) | Detected in small subsets of KP-IR and NKB-IR neurons (present study) | goat anti-human pENK (EB08195; Everest Biotech Ltd) rabbit anti-mENK (IS-30) (Magoul et al., 1993; Covenas et al., 2004) |
| NPY | Not detected in the ARC of mouse (Cravo et al., 2011) | No colocalization in KP and NKB neurons (present study) | sheep anti-NPY (FJL no.14/3A) gift from I. Merchenthaler (Wittmann et al., 2002) |
| NT | Not detected in the RP3V of mouse (Porteous et al., 2011) | No colocalization in KP and NKB neurons (present study) | rat anti-NT (IS-RNT) (Porteous et al., 2011) |
| SP | Not reported | Colocalized in large subsets of KP and NKB neurons (Hrabovszky et al., 2013) | rat anti-SP (8450-0505; AbD Serotec) rabbit anti-SP (S05D3) gift from P. Petrusz (Conti et al., 1992) |
| SS | Not reported | No colocalization in KP and NKB neurons (present study) | guinea pig anti-SS (IS-7/51) (Ciofi et al., 2006) |
| Neurotransmitters | | | |
| TH | Reported in the RP3V of mouse (Clarkson and Herbison, 2011) and rat (Kauffman et al., 2007) | No colocalization in KP and NKB neurons (present study) | chicken anti-TH (TYH; Aves Laboratories) |
| GAD-67 | Reported in both the AVPV and the ARC of mouse (ISH) (Cravo et al., 2011) | Not studied | - |
| vGluT2 | Reported in both the AVPV and the ARC of mouse (ISH) (Cravo et al., 2011) and in the ARC of rat (Ciofi et al., 2006) | Not studied | - |

FIGURE 2 | Summary of neuropeptide/neurotransmitter co-expression data in KP neurons of laboratory animals and the human. AGRP, agouti-related protein; α MSH, α -melanocyte stimulating hormone; ARC, arcuate nucleus; AVPV, anterior periventricular nucleus; CART, cocaine- and amphetamine regulated transcript; CCK, cholecystokinin; Dyn, dynorphin; GAD-67, glutamic acid decarboxylase-67; GAL, galanin; Inf,

infundibular nucleus; ISH, *in situ* hybridization; KP, kisspeptin; mENK, met-enkephalin; NKB, neurokinin B; NPY, neuropeptide Y; NT, neurotensin; pENK, proenkephalin; POMC, proopiomelanocortin; RP3V, rostral periventricular area of the third ventricle; SP, substance P; SS, somatostatin; TH, tyrosine hydroxylase; vGluT2, type-2 vesicular glutamate transporter.

triple-labeled fibers were also detectable in the infundibular stalk, raising the possibility of that these peptides are co-released into the portal circulation. Moreover, some of these axons established occasional contacts with hypophysiotropic GnRH-IR fibers in the postinfundibular eminence, the infundibular stalk and the neurohypophysis (Hrabovszky et al., 2013; Borsay et al., 2014). These anatomical observations suggest that SP may modulate the secretion of KP and NKB via autocrine/paracrine mechanisms and/or act on hypophysiotropic GnRH axons to regulate GnRH release directly.

Cocaine- and amphetamine-regulated transcript (CART)

CART has been implicated in the regulation of metabolic and neuroendocrine processes including reproduction (Rogge et al., 2008; Smith et al., 2010). In rodents, CART-IR fibers form close contacts with GnRH and KP neurons (Leslie et al., 2001; Rondini et al., 2004; True et al., 2013). Both KP and GnRH neurons respond to CART with depolarization. These data suggest that CART influences GnRH neuronal functions directly as well as indirectly, via modulating KP/KISS1R-signaling to GnRH cells (Roa and Herbison, 2012; True et al., 2013).

Colocalization studies of histological specimens from postmenopausal women provided evidence that KP-IR and NKB-IR perikarya often exhibit CART immunoreactivity (Skrapits et al., 2014); 48% of KP-IR and 30% of NKB-IR cell bodies were also CART-IR and 24% of labeled perikarya contained all three signals (Figure 1E). The colocalization phenomena were also detected at the level of fiber varicosities; 17% of KP-IR and 6% of NKB-IR fiber varicosities exhibited CART signal and 5% of all encountered varicosities were triple-labeled (Skrapits et al., 2014) (Figure 1F). Finally, some CART-containing KP and NKB fibers were also immunopositive for SP, indicating the overlap between the CART-IR and the SP-IR populations of KP and NKB neurons (Skrapits et al., 2014).

NEUROPEPTIDES DETECTED AT LOW LEVELS IN HUMAN KP CELLS

Dynorphin (Dyn)

In ISH studies, *Dyn* expressing cells show a similar postmenopausal hypertrophy as do KP and NKB neurons in the Inf (Rometo and Rance, 2008). However, unlike in rodents and sheep where *Dyn* was detected in the vast majority of ARC KP cells, morphological studies from our laboratory only found poor evidence for the presence of *Dyn* immunoreactivity in KP neurons of young men (Hrabovszky et al., 2012) (Figure 1C). Here we have replicated these IHC colocalization experiments on histological samples obtained from postmenopausal women. These new studies were unable to reveal significant amounts of *Dyn* signal in KP-IR neurons or their processes, although scattered dual-labeled fibers were clearly detectable.

Proenkephalin (pENK)/met-enkephalin (mENK)

As part of an immunofluorescent screening for additional neuropeptides in human KP cells, we have addressed the putative colocalization between pENK and KP. To simultaneously visualize KP, NKB, and pENK with triple-labeling, a rabbit KP antiserum (Antibody Verify, Las Vegas, NV USA; AAS26420C; 1:1000), a mouse monoclonal NKB antibody (Biosensis Pty. Ltd, Thebarton, SA Australia; M-871-100; 1:3000) and an affinity-purified goat

pENK antibody (Everest Biotech Ltd, Ramona CA USA; EB08195; 1:1000) were used. Tissue processing and IHC procedures were adapted from similar studies (Hrabovszky et al., 2010, 2011, 2012, 2013; Molnar et al., 2012; Skrapits et al., 2014).

While the confocal analysis provided no evidence for pENK expression in KP and NKB neurons of postmenopausal women ($n = 3$) being in line with a previous observation made in mice (Porteous et al., 2011), small subsets of KP-IR and NKB-IR neurons co-expressed pENK immunoreactivity (Figures 1H,I) in histological samples of human males ($n = 3$; age: 51, 64, and 78 years). The analysis of 212 KP and/or NKB perikarya established the presence of pENK signal in $12.5 \pm 5.1\%$ of NKB-IR and $1.9 \pm 1.0\%$ of KP-IR neurons. Co-expression was also studied in 700 axon varicosities with an approach adapted from our recent study (Skrapits et al., 2014). pENK signal was contained in $5.7 \pm 2.5\%$ of NKB-IR and $4.9 \pm 1.8\%$ of KP-IR axon varicosities. To confirm this colocalization phenomenon, the immunofluorescent detection of pENK in KP neurons has been replicated with a polyclonal rabbit mENK antiserum (IS-30; 1:1000) (Magoul et al., 1993), used in combination with a sheep polyclonal KP antibody (GQ2; 1:1000) (Dhillon et al., 2005) for dual-labeling (Figure 1J).

NEUROPEPTIDES STUDIED BUT NOT DETECTED IN HUMAN KP CELLS

The neurochemical phenotype of KP and NKB neurons was addressed in a large series of IHC experiments using antibodies against further neuropeptide targets. These colocalization studies failed to reveal galanin in human KP and NKB neurons (Figure 1G). This observation reveals a species difference from mice whose KNDy neurons express galanin mRNA and immunoreactivity (Kallo et al., 2012). In line with previous observations made in mice or sheep (Goodman et al., 2007; Cravo et al., 2011; Porteous et al., 2011), agouti-related protein, neuropeptide Y, α -melanocyte-stimulating hormone, neurotensin and cholecystokinin were not detectable in human KP and NKB neurons and these neurons were also immunonegative for somatostatin. Finally, human KP and NKB cells did not contain the dopaminergic marker tyrosine hydroxylase which was colocalized earlier with KP in the RP3V of rodents (Kauffman et al., 2007; Clarkson and Herbison, 2011).

CONCLUSIONS

The functional significance of neuropeptide co-transmitters in KP neurons may vary largely. So far, NKB has been the most consistently detected co-transmitter of KP in the mediobasal hypothalamus, independently of species. Its critical involvement in the regulation of human reproduction is well established by the hypogonadotropic hypogonadism of the *TAC3*- or *TAC3R*-mutant humans (Topaloglu et al., 2009). In recent clinical studies, NKB/NK3R signaling was found essential during early sexual development and its importance became attenuated over time in sustaining the normal functioning of the reproductive axis (Gianetti et al., 2010). It is possible that compensatory mechanisms involve SP which is co-expressed in human KP cells (Hrabovszky et al., 2013) and other neurokinin receptors (NK1R, NK2R) that might substitute NK3R in human KP neurons. A second intensely studied neuropeptide, *Dyn*, is colocalized in the vast majority of KP neurons in rodents, sheep and goats, giving

rise to the KNDy neuron terminology (Lehman et al., 2010b) and single-neuron models of the GnRH/LH pulse generator (Navarro et al., 2009; Ohkura et al., 2009; Wakabayashi et al., 2010). Somewhat surprisingly, our IHC studies did not reveal significant Dyn expression in KP neurons of young men (Hrabovszky et al., 2012) or postmenopausal women (present study). The low level of colocalization challenges the universal importance of endogenous Dyn in the regulation of episodic GnRH/LH secretion by KP neurons. The possibility exists that pENK/mENK we detected in human KP cells replaces some of the functions that Dyn plays in KP cells of laboratory species. The detection of SP, CART and pENK/mENK in KP neurons of the human which was not reported earlier in laboratory species indicates that humans and laboratory animals may use considerably different neuropeptide signaling mechanisms to regulate sex steroid feedback and the GnRH neurosecretory pulses. Finally, a large set of neuropeptides we have tested in this study for co-expression with KP do not seem to be present in KP neurons of any species studied so far. This peptide group includes neurotensin, cholecystokinin, proopiomelanocortin-derivatives, agouti-related protein, neuropeptide Y and somatostatin. Information about the neurochemical phenotype of human KP neurons summarized in this minireview will help us understand the peptidergic regulatory mechanisms of sex steroid feedback and episodic GnRH/LH secretion.

AUTHOR CONTRIBUTIONS

Katalin Skrapits, Philippe Ciofi, Zsolt Liposits and Erik Hrabovszky conceived and designed the experiments and wrote the paper. Beáta Á. Borsay and László Herczeg contributed essential research material.

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Divergent cardio-ventilatory and locomotor effects of centrally and peripherally administered urotensin II and urotensin II-related peptides in trout

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The urotensin II (Ull) gene family consists of four paralogous genes called Ull, Ull-related peptide (URP), URP1 and URP2. Ull and URP peptides exhibit the same cyclic hexapeptide core sequence (CFWKYC) while the N- and C-terminal regions are variable. Ull, URP1, and URP2 mRNAs are differentially expressed within the central nervous system of teleost fishes, suggesting that they may exert distinct functions. Although the cardiovascular, ventilatory and locomotor effects of Ull have been described in teleosts, much less is known regarding the physiological actions of URPs. The goal of the present study was to compare the central and peripheral actions of picomolar doses (5–500 pmol) of trout Ull, URP1, and URP2 on cardio-ventilatory variables and locomotor activity in the unanesthetized trout. Compared to vehicle, intracerebroventricular injection of Ull, URP1 and URP2 evoked a gradual increase in total ventilation (V_{TOT}) reaching statistical significance for doses of 50 and 500 pmol of Ull and URP1 but for only 500 pmol of URP2. In addition, Ull, URP1 and URP2 provoked an elevation of dorsal aortic blood pressure (P_{DA}) accompanied with tachycardia. All peptides caused an increase in locomotor activity (A_{CT}), at a threshold dose of 5 pmol for Ull and URP1, and 50 pmol for URP2. After intra-arterial (IA) injection, and in contrast to their central effects, only the highest dose of Ull and URP1 significantly elevated V_{TOT} and A_{CT} . Ull produced a dose-dependent hypertensive effect with concomitant bradycardia while URP1 increased P_{DA} and heart rate after injection of only the highest dose of peptide. URP2 did not evoke any cardio-ventilatory or locomotor effect after IA injection. Collectively, these findings support the hypothesis that endogenous Ull, URP1 and URP2 in the trout brain may act as neurotransmitters and/or neuromodulators acting synergistically or differentially to control the cardio-respiratory and locomotor systems. In the periphery, the only physiological actions of these peptides might be those related to the well-known cardiovascular regulatory actions of Ull. It remains to determine whether the observed divergent physiological effects of Ull and

URPs are due to differential interaction with the UT receptor or binding to distinct UT subtypes.

Keywords: urotensin II, urotensin II-related peptides, ventilatory variables, heart rate, blood pressure, locomotor activity, brain, trout

Introduction

Urotensin II (UII) is a cyclic neuropeptide that was originally isolated and purified from the caudal neurosecretory system of the teleost fish *Gillichthys mirabilis* (longjaw mudsucker) on the basis of its smooth muscle-stimulating activity (Bern and Lederis, 1969; Pearson et al., 1980). Recently, it has been demonstrated that UII belongs to a family of structurally related peptides that include UII and UII-related peptides (URPs) called URP, URP1, and URP2 (Lihrmann et al., 2013). UII, URP, URP1, and URP2 exhibit the same cyclic hexapeptide core sequence (CFWKYC) while the N- and C-terminal regions are variable (Conlon, 2008; Lihrmann et al., 2013). In the teleost lineage, all four paralog genes are present but only two of them, UII and URP, are found in tetrapods (Quan et al., 2012; Tostivint et al., 2013). In mammals, UII and URP genes are mostly expressed in cholinergic neurons of the brainstem and spinal cord but variable levels of expression occur in most brainstem nuclei (Vaudry et al., 2015) suggesting that the peptides may exert distinct biological functions. UII and URP mRNAs are also differentially expressed in peripheral tissues, including notably the cardiovascular, renal and endocrine systems (Sugo et al., 2003; Dubessy et al., 2008; Vaudry et al., 2015). UII and URP both activate the UT receptor with the same potency (Sugo et al., 2003) but the two peptides may exert differential modulatory effects due to recruitment of different intracellular signaling pathways (Vaudry et al., 2010). The UT receptor is present in several areas of the brain and spinal cord but also in various peripheral organs including the cardiovascular system, endocrine tissues and kidney (Vaudry et al., 2015). UII exerts a large array of biological effects including regulation of various behaviors, motor and neuroendocrine activities, as well as central and peripheral control of blood pressure and heart rate but much less is known about the biological actions of URPs (Vaudry et al., 2010, 2015). In fish, UII, URPs and the UT receptor are also present in the brain and spinal cord. Pioneer studies have demonstrated that UII-like immunoreactivity is primarily found in cerebrospinal fluid (CSF)-contacting neurons located within the ventral ependyma lining the central canal along the entire length of the spinal cord and the medulla oblongata (Yulis and Lederis, 1986, 1988). These CSF-contacting neurons containing UII-like immunoreactivity project their axons toward the external surface of the spinal cord and ascending fibers innervate various regions of the brain (Yulis and Lederis, 1986, 1988). UII has been purified and characterized from extracts of the brains of an elasmobranch, the skate *Raja rhina*, and a teleost,

the rainbow trout *Oncorhynchus mykiss* (Waugh and Conlon, 1993). The expression of UII mRNA in fish brain has been confirmed by RT-PCR in the European flounder *Platichthys flesus* (Lu et al., 2006), in the zebrafish *Danio rerio* (Parmentier et al., 2008) and in the orange-spotted grouper *Epinephelus coioides* (Sun et al., 2014). Extensive studies on the differential expression of URP, URP1 and URP2 in the central nervous system (CNS) have been conducted in the Japanese eel *Anguilla japonica* (Nobata et al., 2011) and in the zebrafish (Parmentier et al., 2011; Quan et al., 2015). In zebrafish, URP mRNA is present in motoneurons (cited in Quan et al., 2015). In both species, URP1 is mainly expressed in motoneurons of the medulla oblongata. In zebrafish, URP2 mRNA is found in cells located along the ventral edge of the fourth ventricle, probably in CSF-contacting neurons, and in the spinal cord, URP1 and URP2 mRNAs co-localize in same cells that are also CSF-contacting neurons (Quan et al., 2015). In the flounder (Lu et al., 2006), the killifish *Fundulus heteroclitus* (Evans et al., 2011) and the orange-spotted grouper (Sun et al., 2014), the UT receptor is strongly expressed in the caudal neurosecretory system, the CNS (brain and spinal cord) but also in various peripheral tissues including the heart, gill, kidney and ovary. UII is known to be involved in osmoregulation in fish (Marshall and Bern, 1979; Lu et al., 2006; Evans et al., 2011) and a few studies have examined the cardiovascular effects of UII and URP1 in teleosts. In the rainbow trout, centrally administered UII evokes an increase in dorsal aortic blood pressure (P_{DA}) with variable action on the heart (Le Mével et al., 1996), while intra-arterial (IA) injection of UII provokes a dose-dependent elevation in P_{DA} with a concomitant bradycardia (Le Mével et al., 1996). In the Japanese eel, the cardiovascular effects of centrally and peripherally injected UII and URP1 are quite similar. Both peptides preferentially elevate blood pressure in the ventral aorta than in the dorsal aorta and evoke tachycardia (Nobata et al., 2011). In addition, in the rainbow trout, central injection of UII produces a hyperventilatory response and a long-lasting increase in locomotor activity (Lancien et al., 2004). Nonetheless, due to the relatively recent discovery of URPs, nothing is known about the potential actions of URP1 on ventilatory and locomotor functions and those of URP2 on cardio-ventilatory and locomotor functions. The differential although similar expression of UII, URP1, and URP2 in the CNS of teleosts suggests that these peptides may have synergistic or divergent biological effects. It is thus important to determine the *in vivo* integrative actions of these peptides on physiological functions and behavior in the same animal. Therefore, the main goal of the present study was to analyze the central effects of trout UII, URP1, and URP2 on ventilatory and cardiovascular functions and on locomotor activity in our established trout model. To this end, we have analyzed the effects of intracerebroventricular (ICV) administration of synthetic replicates of these peptides on ventilatory amplitude (V_{AMP}), ventilatory frequency (f_v), total ventilation (V_{TOT}), P_{DA} ,

Abbreviations: A_{CT} , locomotor activity; a.u., arbitrary unit; CNS, central nervous system; ECG, electrocardiographic; f_H , heart rate; f_v , ventilatory rate; IA, intra-arterial; ICV, intracerebroventricular; P_{DA} , dorsal aortic blood pressure; UII, urotensin II; URP, urotensin II-related peptide; URP1, urotensin II-related peptide 1; URP2, urotensin II-related peptide 2; V_{AMP} , ventilatory amplitude; V_{TOT} , total ventilation.

heart rate (f_H), and locomotor activity (A_{CT}). Additionally, the central actions of the peptides were also compared with their effects after IA injection.

Material and Methods

Peptides and Chemicals

The primary sequence of the urotensin peptides examined in this study is shown in **Table 1**. Trout UII, URP1, and URP2 (Vaugh and Conlon, 1993; Tostivint et al., 2013) were synthesized as previously described (Chatenet et al., 2004; Lancien et al., 2004). The peptides were dissolved in Ringer’s solution (vehicle) and stored in stock solutions at -25°C . Immediately before use, UII, URP1, or URP2 were diluted to the desired concentration with Ringer’s solution. The composition of the Ringer’s solution was (in mM): NaCl 124, KCl 3, CaCl_2 0.75, MgSO_4 1.30, KH_2PO_4 1.24, NaHCO_3 12, glucose 10 (pH 7.8). All solutions were sterilized by filtration through $0.22\text{ }\mu\text{m}$ filters (Millipore, Molsheim, France) before injection.

Animals

Adult rainbow trout *Oncorhynchus mykiss* ($247 \pm 24\text{ g}$ body wt, mean \pm SEM, $n = 95$) of both sexes were purchased locally and transferred in a well-oxygenated and thermostatically controlled water tank to the laboratory. All fish were kept in a 1000-liter tank containing circulating dechlorinated and aerated tap water ($11\text{--}12^{\circ}\text{C}$), under a standard photoperiod (lights on 09:00–20:00). The fish were allowed at least 3 weeks to acclimate under these conditions before the experiments were started. Experimental protocols were approved by the Regional Ethics Committee in Animal Experiments of Brittany, France.

Experimental Procedures

All surgical procedures were made under tricaine methane-sulfonate (3-aminobenzoic acid ethyl ester methanesulfonate; 60 mg/L in tap water buffered with NaHCO_3 to pH 7.3–7.5) anesthesia. The techniques used for placement of the electrocardiographic (ECG) electrodes, placement of the buccal catheter, cannulation of the dorsal aorta and insertion of the ICV microguide have previously been described in detail (Le Mével et al., 1993; Lancien et al., 2004). Briefly, two ECG AgCl electrodes (Compea, Bagnolet, France) were subcutaneously implanted ventrally and

longitudinally at the level of the pectoral fins. The incision was sutured across the electrodes and the leads were sutured to the skin. The dorsal aorta was cannulated with a PE-50 catheter (Clay Adams, Le Pont De Claix, France). A flared cannula (PE-160) was inserted into a hole drilled between the nares such that its flared end was resting against the roof of the mouth. This cannula was used to record any changes in buccal ventilatory pressure. The absence of a neocortex in fish allows the accurate placement of the ICV microguide under stereomicroscopic guidance. A 25-gauge needle fitted with a PE-10 polyethylene catheter was inserted between the two habenular ganglia and descended into the third ventricle until its tip lay between the two preoptic nuclei (Le Mével et al., 2009). An obturator was placed at the end of the PE-10 tubing and the cranial surface was covered with hemostatic tissue followed by light quick-curing resin. After surgery, the animals were force-ventilated with dechlorinated tap water until recovery of opercular movements and transferred to a 6-liter blackened chamber supplied with dechlorinated and aerated tap water ($10\text{--}11^{\circ}\text{C}$) that was both re-circulating and through-flowing. Oxygen partial pressure within the water tank (P_{wO_2}) and pH were continuously recorded and maintained at constant levels ($P_{\text{wO}_2} = 20\text{ kPa}$; pH = 7.4–7.6). A small horizontal aperture was made along the upper edge of the chamber in order to connect the ECG leads to an amplifier and to connect the dorsal aorta and the buccal cannula to pressure transducers. This aperture also permitted ICV and IA injections of peptides without disturbing the animals.

Trout were allowed to recover from surgery and to become accustomed to their new environment for 48–72 h. Each day, the general condition of the animals was assessed by observing their behavior, checking the ventilatory and the cardiovascular variables, and measuring their hematocrit. Animals that did not appear healthy, according to the range of values detailed in our previous studies, were discarded. After stable V_{AMP} , f_V , P_{DA} , and f_H were maintained for at least 90 min, parameters were recorded for 30 min without any manipulation in control experiments. To minimize the use of experimental animals, some trout received both ICV and IA injections. In this later case, the delay between the two injections was 1 day, and the order of the injections was randomized among animals. No single fish was studied for more than 2 days and control experiments revealed that there was no significant change in performance over this period.

Intracerebroventricular Administration of Peptides

The injector was introduced within the ICV guide prior to the beginning of a recording session which lasted 30 min. All injections were made at the fifth minute of the test but the injector was left in place for a further 5 min to allow for complete diffusion of the agent and to minimize the spread of substances upwards in the cannula tract. The fish received first an ICV injection of vehicle ($0.5\text{ }\mu\text{l}$) and 30 min later, an ICV injection of UII, URP1, or URP2 (5, 50, and 500 pmol in $0.5\text{ }\mu\text{l}$). The rationale for using these doses was that they were in the same range as those previously used for studies on the cardiovascular effects of UII in trout and for comparison of effects between peptides (Le Mével et al., 1996, 2012). Previous control experiments using two ICV

TABLE 1 | Amino-acid sequence of trout urotensin II (Vaugh and Conlon, 1993) and teleost URP1 and URP2 (Tostivint et al., 2013) examined for their cardio-ventilatory effects and locomotor activity following central and peripheral injection in the unanesthetized rainbow trout *Oncorhynchus mykiss*.

| Urotensin II peptides | Amino-acid sequence |
|-----------------------|---|
| UII | H-Gly-Gly-Asn-Ser-Glu- Cys-Phe-Trp-Lys-Tyr-Cys -Val-OH |
| URP1 | H-Ala- Cys-Phe-Trp-Lys-Tyr-Cys -Val-Thr-Asn-OH |
| URP2 | H-Val- Cys-Phe-Trp-Lys-Tyr-Cys -Ser-Gln-Asn-OH |

The conserved cyclic hexapeptide core sequence (Cys-Phe-Trp-Lys-Tyr-Cys or CFWKYC) of each peptide is in bold characters.

injections 30 min apart have shown no time-dependent changes in the measured variables using this protocol (Le Mével et al., 2009). The animals received no more than two ICV injections of peptide per day with a delay of at least 5 h between the injections.

Intra-Arterial Administration of Peptides

Five minutes after the beginning of the recording session, 50 μ l of vehicle, UII, URP1, or URP2 at doses of 5, 50, and 500 pmol was injected through the dorsal aorta and immediately flushed by 150 μ l of vehicle.

Data Acquisition and Analysis of Cardio-Ventilatory Variables and Motor Activity

The ECG electrodes were connected to a differential amplifier (band pass: 5–50 Hz; Bioelectric amplifier, Gould & Nicolet, Courtaboeuf, France) and a stainless steel bar was immersed in the water of the tank to act as a reference electrode. The aortic cannula and the buccal catheter were connected to P23XL pressure transducers (band pass: 0–15 Hz; Gould & Nicolet). These pressure transducers were calibrated each day using a static water column. At the beginning of the experiments, the zero-buccal pressure level was set electronically. The output signals from the devices were digitalized at 1000 Hz and visualized on the screen of a PC using PowerLab 4/30 data acquisition system (ADInstruments, Oxford, England) and LabChart Pro software (v.7.0; ADInstruments, Oxford, England) during the 30-min recording period and the data were stored on a disk. The time-series related to the ventilatory, the pulsatile P_{DA} and the ECG signals were then processed off-line with custom-made programs written in LabView 6.1 (Laboratory Virtual Instrument Engineering Workbench, National Instruments, Austin, USA). Motor activity, ventilatory and cardiovascular variables were calculated as previously described (Lancien et al., 2004; Le Mével et al., 2007). Motor activity was detected as artifacts on the ventilatory signal (Lancien et al., 2004) and the total duration of locomotor activity (A_{CT} , in seconds) was determined from ventilatory signal (Lancien et al., 2004). Thereafter, segments free of any movement artifacts on the ventilatory signal were selected and f_V (breaths min^{-1}) and V_{AMP} (arbitrary units, a.u.) were determined. The f_V was calculated from the first harmonic of the power spectrum of the ventilatory signal using the fast Fourier transformation. V_{AMP} was calculated from the difference between the maximal abduction phase and the maximal adduction phase for each ventilatory cycle. Spontaneous coughings, which correspond to rapid and robust changes in the abduction/adduction phases of the ventilatory cycle, were excluded from this analysis. The net effect of the changes in f_V and V_{AMP} on ventilation was estimated according to the formula $V_{TOT} = f_V \times V_{AMP}$, where V_{TOT} (a.u.) is total ventilation. Mean P_{DA} (kPa) was calculated from the pulsatile P_{DA} as the arithmetic mean between systolic blood pressure and diastolic blood pressure, and the f_H (beats min^{-1}) was determined from the ECG signal. All calculations for mean f_V , V_{AMP} , V_{TOT} , P_{DA} , f_H , and A_{CT} were made for the pre-injection period (0–5 min) and for five post-injection periods of 5 min for each trout. To reduce the amount of data, only the maximal effects of the various treatments in the above parameters were analyzed and the results were averaged for trout subjected to the same

protocol. One-Way ANOVA analysis of baseline values of f_V , V_{AMP} , V_{TOT} , P_{DA} , f_H , and A_{CT} during the pre-injection period revealed that there was no statistical difference between groups prior ICV or IA injection of vehicle or the various peptides (not shown).

Statistical Analysis

Data are expressed as means + SEM (standard error of the mean). The data were analyzed by One-Way ANOVA test followed by the multiple comparison tests of Dunnett or Tukey. The criterion for statistical difference between groups was $P < 0.05$. The statistical tests were performed using GraphPad Prism 5.0 (GraphPad, San Diego, USA).

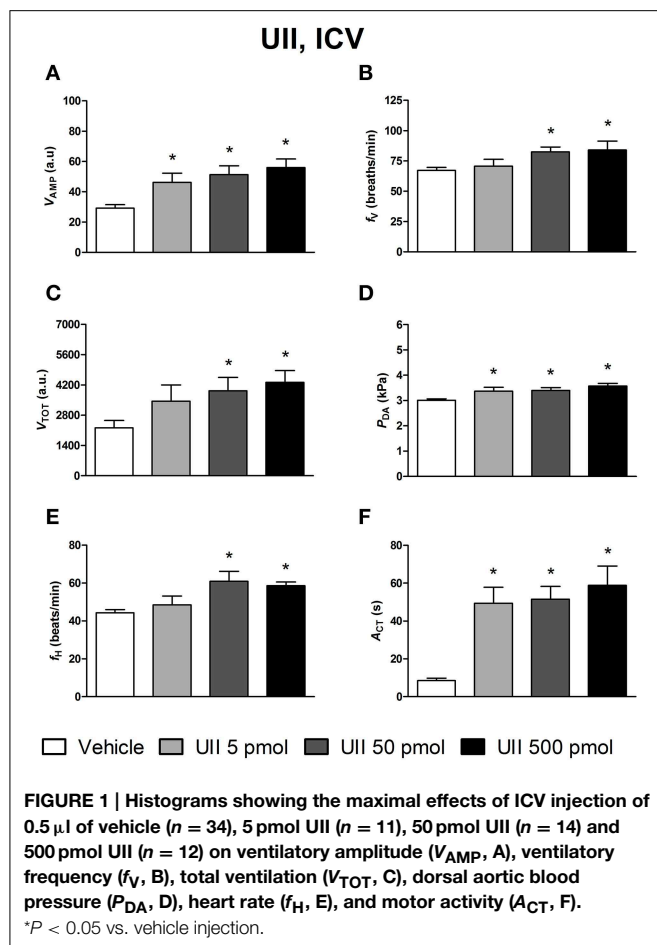
Results

Ventilatory, Cardiovascular and Locomotor Activity Responses to Central UII, URP1, and URP2

The effects of ICV injections of vehicle, UII, URP1, and URP2 on ventilatory and cardiovascular variables, and on locomotor activity are summarized in **Figures 1–3**, respectively. Compared with ICV injection of vehicle, all peptides evoked quite similar increase in V_{AMP} (**Figures 1A, 2A, 3A**) and f_V (**Figures 1B, 2B, 3B**). However, the threshold dose of UII inducing a significant effect on V_{AMP} was only 5 pmol while a 10-fold higher dose was required for URP1 and URP2. All peptides provoked an elevation of f_V for a threshold dose of 50 pmol with minor differences between peptides at the higher dose (**Figures 1B, 2B, 3B**). The net effect of the ICV administration of the peptides was a hyperventilatory response involving a gradual and significant increase in V_{TOT} for doses of 50 and 500 pmol of UII and URP1 but for only 500 pmol of URP2 (**Figures 1C, 2C, 3C**). In addition, UII, URP1, and URP2 provoked a non-dose-dependent increase in P_{DA} (**Figures 1D, 2D, 3D**). However, the threshold dose for this effect was only 5 pmol for UII and URP1 (**Figures 1D, 2D**) but 500 pmol for URP2 (**Figure 3D**). During this hypertensive effect of the peptides, there was no bradycardia but instead, a significant tachycardia occurred at the 50 and 500 pmol doses for most of the peptides (**Figures 1F, 2F, 3F**). UII, URP1, and URP2 also caused a potent increase in A_{CT} for a threshold dose of 5 pmol for UII and URP1 but 50 pmol for URP2.

Ventilatory, Cardiovascular and Locomotor Activity Responses to Peripheral UII, URP1, and URP2

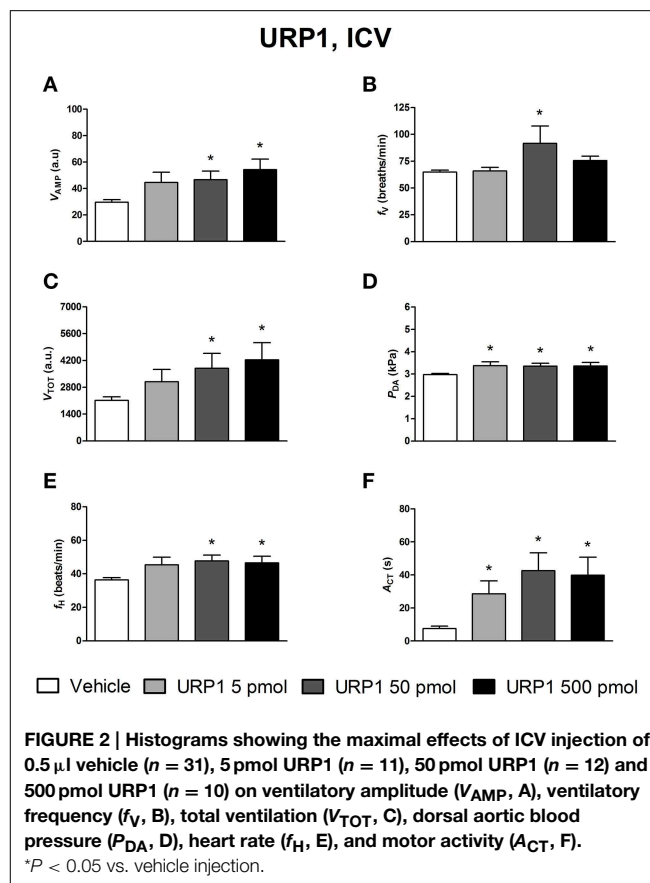
Figures 4–6 depict the results obtained after IA administration of the different peptides on ventilatory and cardiovascular variables and on motor activity. In contrast to their central effects, only the highest dose of UII and URP1 (500 pmol) significantly elevated V_{AMP} and the net effect of these peptides was a hyperventilatory response since V_{TOT} significantly increased (**Figures 4C, 5C**). Contrary to its ICV effects, IA injection of UII produced a significant dose-dependent increase in P_{DA} (**Figure 4D**) accompanied with a f_H decrease, a bradycardia statistically significant for the 5 and 50 pmol doses of peptide (**Figure 4E**). Only the highest dose of URP1 (500 pmol) provoked



an elevation in P_{DA} accompanied by a significant tachycardia (Figures 5D,E). IA injection of the highest dose of UII and URP1 caused an increase in A_{CT} (Figures 4F,5F). Peripheral administration of URP2 at any dose did not produce any effect on the cardio-ventilatory variables and locomotor activity (Figures 6A–F).

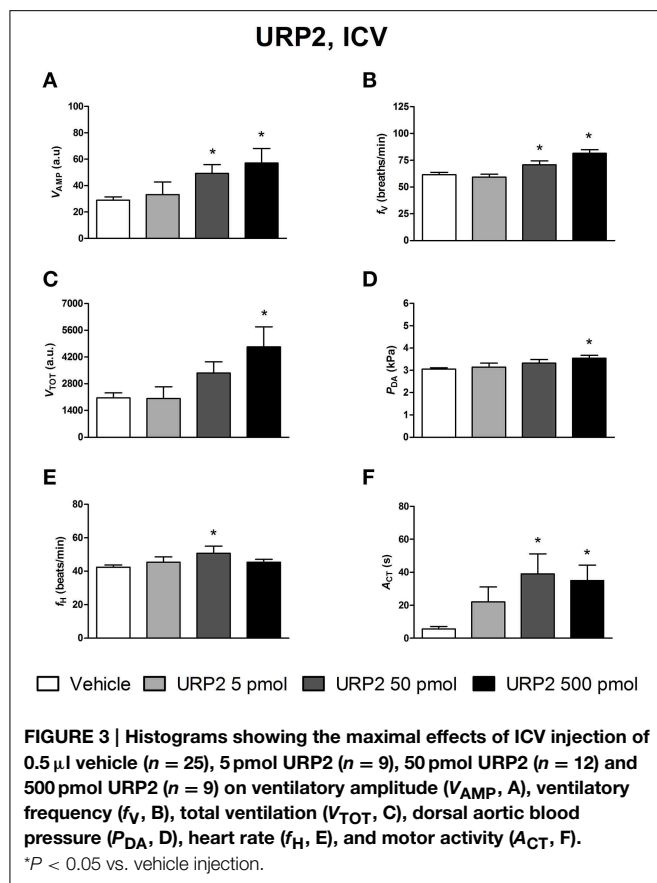
Discussion

This is the first functional study evaluating the integrative effects of UII, URP1, and URP2 on physiological variables including ventilation, blood pressure and locomotor activity in fish. The most important outcome of this study is that ICV or IA administration of picomolar doses of these peptides exert both common and specific biological activities depending on the route of administration. The demonstration that ICV injection of UII and URP1 evoked a stimulatory action on ventilation, cardiovascular variables and locomotion for doses that did not produce any effect or evoked differential action after peripheral administration, supports the assumption that following ICV injection, central neuronal sites are involved in the action of the peptides. In addition, after IA injection, the exclusive effect of low picomolar doses of UII on P_{DA} and f_H , confirms that this peptide may act also to peripheral sites.



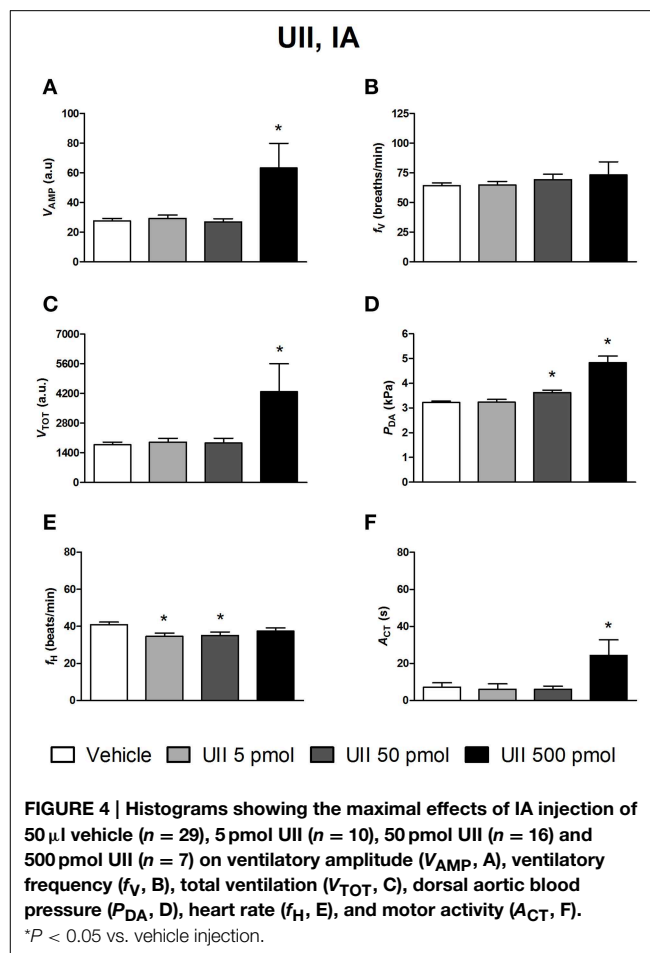
Ventilatory, Cardiovascular and Locomotor Actions of Centrally Administered UII, URP1, and URP2

The central actions of UII, URP1, and URP2 on cardio-ventilatory and motor functions may be compared to those reported in previous studies conducted with UII or URP1 in fish or in other vertebrates species. The present results on UII are consistent with our previous data demonstrating that, in trout, UII administered through the ICV route causes a non-dose-dependent elevation of P_{DA} without bradycardia (Le Mével et al., 1996). In the eel, central administration of UII and URP1 preferentially elevates blood pressure of the branchial circulation higher than that of the systemic circulation (Nobata et al., 2011). Consequently, the hypertensive effect of eel UII on ventral aortic blood pressure (P_{VA}) is significant for doses higher than 0.15 nmol while doses higher than 0.5 nmol are required to increase P_{DA} . URP1 (0.3–0.5 nmol) provokes a significant dose-dependent increase in P_{VA} , but does not affect P_{DA} at any dose (Nobata et al., 2011). The effect of URP1 was longer lasting than that of UII and the two peptides evoked tachycardia (Nobata et al., 2011). The absence of bradycardia in response to an increase in blood pressure following ICV injection of UII and URP1 in trout and eel, and URP2 in trout suggests that the cardio-inhibitory baroreceptor reflex is altered following central injection of these peptides. In normotensive and hypertensive unanesthetized rats (Lin et al., 2003a,b) and in unanesthetized sheep (Watson and May,



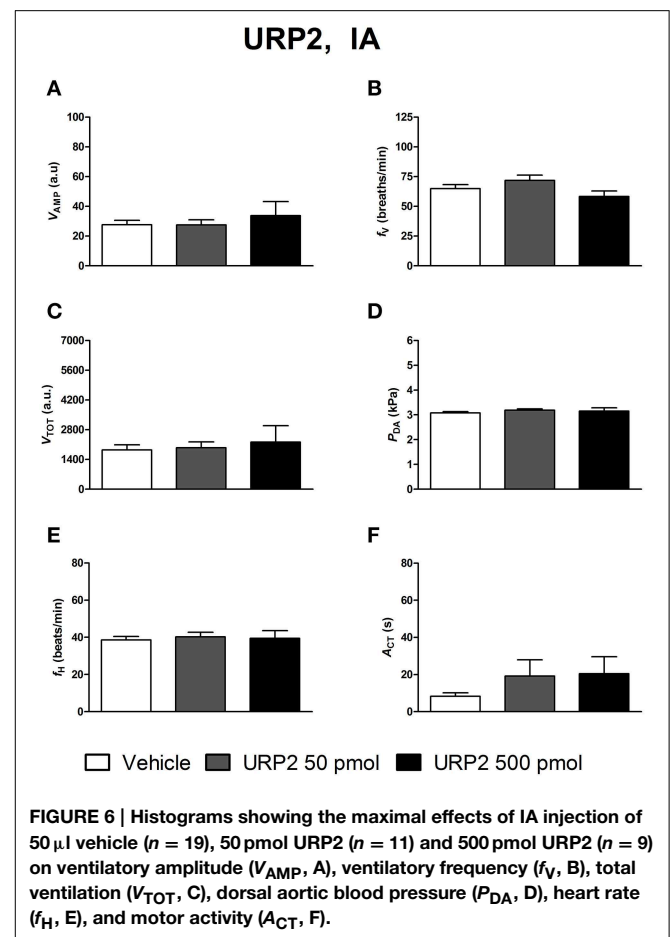
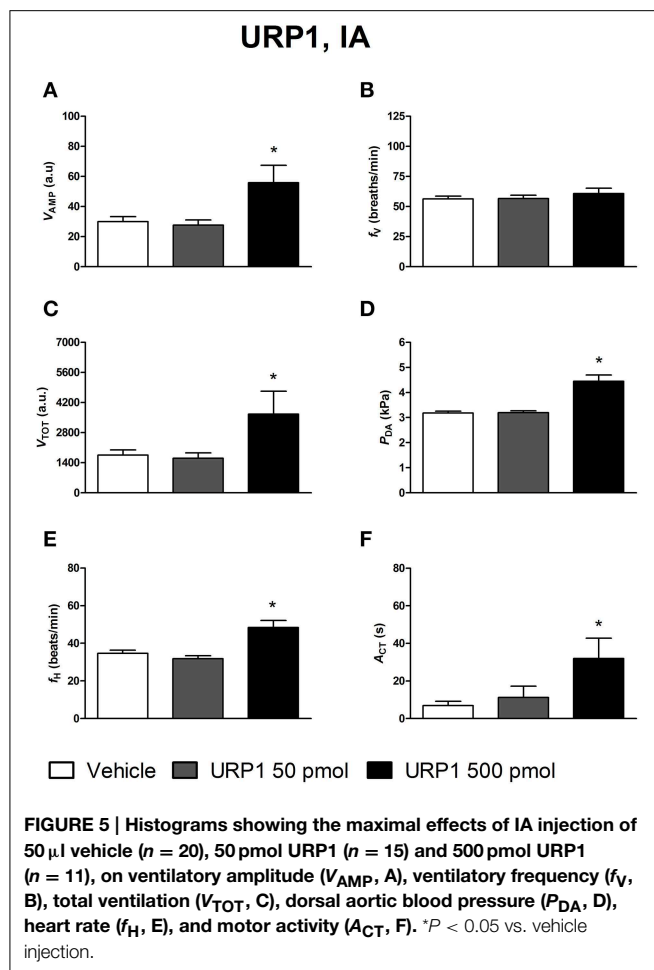
2004), ICV administration of UII causes pressor and tachycardic responses through activation of the sympathetic system indicating that, in these species, also the cardiac baroreflex response is impaired. Studies conducted on unanesthetized sheep to test this hypothesis demonstrated that, after ICV infusion of UII (0.2 nmol/kg for 1 h), the cardiac baroreflex response is effectively blunted since no changes occur in the cardiac sympathetic nerve activity in spite of an increase in blood pressure (Hood et al., 2005). In rats, the central cardiovascular action of UII is site-dependent and local administration of UII in discrete brain nuclei produces differential cardiovascular responses (Lu et al., 2002). To our knowledge, the central action of URP in mammals has never been explored.

We have previously demonstrated that, in addition to its central cardiovascular effects, UII produces a hyperventilatory response and a stimulatory effect on locomotion (Lancien et al., 2004, 2005). In the present study, UII-induced hyperventilation was mimicked by URP1 and to a lesser extent by URP2. Furthermore, at the low dose of 5 pmol, UII, URP1 but not URP2 provoked an increase in locomotion. Nonetheless, at this picomole dose, UII and URP1 did not induced any change in V_{TOT} . These observations suggest that UII and URP1 act preferentially on central neuronal networks controlling locomotion than ventilation. This stimulatory effect of UII on locomotor activity in fish is in accord with results obtained in rats (Gartlon et al., 2001) and mice (Do-Rego et al., 2005) showing that ICV injection of



human UII (hURP, ACFWKYCV) and mouse UII, respectively, elicit motor activity in a familiar environment. It should be noted, however, that the threshold doses eliciting locomotor effects in rats and mice are in the nanomole range. Because UII and URPs induced a marked increased in locomotor activity in trout, we cannot exclude that the changes observed in cardio-ventilatory parameters may be secondary effects of the peptides. Finally, in our study and after ICV injection, a trend in the potency order of UII, URP1, and URP2 emerged being $UII \geq URP1 > URP2$ notably for the hyperventilatory, hypertensive and locomotor actions of these peptides.

The receptor site(s) and the multisynaptic pathways involved in initiating cardio-ventilatory and locomotor responses after UII, URP1, and URP2 injection within the brain are matter of speculation and require further studies. Nevertheless, as previously mentioned for the central actions of other neuropeptides, some neuroanatomical prerequisites and some neurophysiological data exist that may support some working hypothesis (Le Mével et al., 2012). Because the peptides were injected within the third ventricle in close proximity to the preoptic nucleus (NPO), they can activate these preoptic neurons leading to hyperventilatory and hypertensive responses through neuroendocrine and/or neurogenic pathways. Preoptic neurons synthesize the nonapeptides vasotocin (AVT) and isotocin (IT). AVT and IT



neurons project not only to the neurohypophysis, but also to the brainstem cardiovascular and ventilatory nuclei (Batten et al., 1990; Saito et al., 2004). It should be emphasized that in trout, AVT produces a hypertensive response acting both centrally and peripherally (Le Mével et al., 1993). UII and URPs injected within the third ventricle may also stimulate locomotion through the direct or indirect projection of neurons from the NPO to mid-brain locomotor nuclei (Lancien et al., 2004) or spinal motor neurons as previously suggested for the control of sexual behavior (Demski and Sloan, 1985; Gregory and Tweedle, 1985). In addition, since the peptides are injected within the CSF, they can diffuse to the mid- and hindbrain to affect motor nuclei involved in cardio-ventilatory functions and swimming behavior (see also 23). Interestingly, the presence of immunoreactive UII, and URP2 gene expression, in CSF-contacting neurons in regions surrounding notably the fourth ventricular wall, has already been documented in various teleosts (Yulis and Lederis, 1988; Parmen-tier et al., 2011; Quan et al., 2015), suggesting that these cells may sense the composition of the CSF and/or release their products within the ventricular system. Furthermore, in the eel brainstem, the URP1 gene is detected within neurons of the commissural nucleus of Cajal, a nucleus homologous to the nucleus tractus solitarius, the first central relay in the cardiovascular baroreflex

loop (Nobata et al., 2011). Concurrently, in the zebrafish, URP1-expressing cells are located in the reticular formation and the glosso-pharyngeal-vagal-motor nuclei (Quan et al., 2015). Collectively, our functional study and these neuroanatomical data support a role of endogenous UII, URP1 and URP2 as neurotransmitters or neuromodulators involved in the central command of autonomic cardio-ventilatory and locomotor functions.

Ventilatory, Cardiovascular and Locomotor Actions of Peripherally Administered UII, URP1, and URP2

The peripheral actions of UII, URP1, and URP2 on cardio-ventilatory and locomotor functions may be compared with those reported in previous studies that explored the peripheral effects of UII, URP, or URP1 in fish or in other vertebrate species but also with their central actions. The present results on UII are in line with our previous data obtained in trout demonstrating that low picomole doses of UII cause a dose-dependent hypertensive response and a bradycardia (Le Mével et al., 1996). In addition, we previously investigated the cardiovascular actions of peripherally injected trout UII (50 pmol) and hURP (50 and 500 pmol) in trout (Le Mével et al., 2008). It appears that hURP is about ten times less potent than trout UII in evoking a hypertensive response since hURP (50 pmol, about 0.2 nmol/kg) had no

significant effect on cardiovascular variables and only the highest dose of hURP (500 pmol, about 2 nmol/kg) produced a similar peak increase in P_{DA} . Furthermore, the hypertensive response observed following the IA injection of hURP was of shorter duration than after IA injection of UII and there was no concomitant bradycardia. The cardiovascular effects of UII/URPs in trout are quite different to those obtained in eel, suggesting that the cardiovascular actions of UII/URPs may be species dependent. Indeed, at an equimolar dose of 0.1 nmol/kg in eel, the vasoconstrictor effects of native UII and URP1 are similar, but as for the ICV injection, the effect of UII is longer lasting than the action of URP1. However, in eel, both eel UII and URP1 evoke a tachycardia (Nobata et al., 2011). In addition, after peripheral injections, both hUII and URP are also less potent than the homologous peptides in eel. Thus, the results obtained with heterologous peptides in trout and in eel, emphasize the importance of the amino-acid residues flanking the N-terminus of the cyclic core of the fish UII-molecule in interacting with the fish UT receptor. In trout, the hypertensive effect of UII is mediated through an increase in the systemic vascular resistance since cardiac output decreases (Le Mével et al., 1996). In mammals including humans, a great amount of heterogeneity of vasoactive responses to UII has been observed among vascular beds from species, as well as different regions within the same species (Douglas et al., 2000). The physiological relevance of our experiments may be questioned since the concentration of the injected peptides within the circulation might be more pharmacological than physiological. Consequently, it remains to be determined if physiological plasma concentration of UII may have a role in the cardiovascular regulation in teleosts. In the present study, the highest doses of UII and URP1 but not URP2 evoked cardio-ventilatory and locomotor effects similar to those observed after ICV injection of these peptides. We make the assumption that these effects were mediated through a neurogenic pathway after diffusion of these peptides to critical target sites in the brain that lack the blood-brain barrier (BBB). Some neuroanatomical and functional data favor this hypothesis. At the level of the medulla oblongata, the area postrema is devoid of BBB and acts as a circumventricular organ in the goldfish *Carassius auratus* (Morita and Finger, 1987) and in the eel *Anguilla japonica* (Tsukada et al., 2007). In eel, the organum vasculosum of the lamina terminalis is another circumventricular organ without BBB that may serve as a window for a central action of peripherally injected regulatory peptides (Mukuda et al., 2013). Of interest, this latter organ projects to the NPO. Collectively, these neuro-anatomical data in

fish and mammals are consistent with the view that circulating UII, and eventually URP1 but not URP2, may act also as signaling molecules to command some neurally-mediated regulatory mechanisms and notably cardio-ventilatory but also locomotor outputs.

In mammals, the UT receptor is the only high affinity receptor for UII/URP known so far (Vaudry et al., 2015). The UT receptor in teleosts shares about 60% identity with the human UT receptor and, as previously mentioned, is strongly expressed in the caudal neurosecretory system, the CNS and in various peripheral tissues (Lu et al., 2006; Evans et al., 2011; Sun et al., 2014). However, recent data provide evidence for the existence of a vertebrate ancestral UT gene that possessed five distinct UT subtypes in teleosts (Tostivint et al., 2014). The functional role of these receptor subtypes in physiological regulations is currently unknown. It might be questioned whether the divergent physiological effects of UII and URPs after central and peripheral injection observed in the present study may be due to differential interaction with the UT receptor or binding to distinct UT receptor subtypes.

In conclusion, we have examined for the first time in fish the integrative central and peripheral physiological effects of UII, URP1 and URP2 on cardio-ventilatory and locomotor functions. The principal and novel findings of this study are that all peptides produce a central stimulatory effect on ventilation, blood pressure, heart rate and locomotion but with variable potency among peptides. Since the UII, URP1, and URP2 genes are expressed in the CNS, our results suggest that the endogenous peptides may be implicated as neurotransmitters or neuromodulators in the regulation of cardio-ventilatory and locomotor functions in trout. After systemic administration of low picomole doses, none of the UII and URP peptides affect ventilation or locomotion, but only UII evokes hypertension and bradycardia, indicating that endogenous UII may have a role as circulating hormone involved in cardiovascular regulation in trout. Further studies are clearly required to determine under which circumstances the different neuroendocrine and neuronal pathways that mediate the integrative effects of the urotensineric system are recruited to participate in cardio-ventilatory and locomotor regulations.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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